5

The genetic manipulation of herbicide tolerance

INTRODUCTION

The preceding four chapters have given an overview of the tools of plant biotechnology that can be used for genetic manipulation. The remaining section of the book will look in detail at the major targets for the genetic manipulation of crops and how they are being approached or have been achieved. Thus, each chapter will describe the scientific strategies that could be used to attain a particular goal for crop improvement: why certain genes might be useful, how they might be transformed into plants, and how their expression might be regulated. In addition, some of the wider issues surrounding genetically modified (GM) crops will be explored: why have certain targets received so much commercial attention, which GM strategies have actually been successful in the field, and are the concerns about the safety of GM crops justified?

● Herbicide tolerance is the predominant GM trait.

The first chapter in this sequence of different GM applications deals with the genetic manipulation of herbicide-tolerant plants. Herbicide tolerance was one of the first GM traits to be tested in the field, and subsequently for commercial production. Table 5.1 shows that the greatest number of trial sites set up during the period 1987–1997 were to test crops carrying this trait, and that herbicide-tolerant crops form more than three-quarters of the total released GM crops in terms of area of commercial planting. (Indeed, note that of all the traits described in this book, only

Table 5.1 Most frequent transgenic traits in commercial plantings

Trait	Area of commercial planting (million ha)	
	2000	2005
Herbicide tolerance	32.7 (74%)	63.7 (71%)
Insect resistance	8.3 (19%)	16.2 (18%)
Herbicide tolerance + insect resistance	3.1 (7%)	10.1 (11%)

(Data from James, 2001, 2005, web-link 5.12.)

herbicide tolerance and insect resistance (see Chapter 6) are widely grown, as yet.) One of the aims of this chapter is to explain why there has been such rapid development of this particular trait, and why crops genetically modified to be herbicide tolerant remain the most widely grown.

There are a number of scientific reasons for the rapid development of herbicide-tolerant crops.

There are scientific and commercial reasons why herbicide-tolerant crops have been developed so rapidly compared with other GM traits. The scientific reasons include the following.

1 Considerable information was already available about the modes of action of certain herbicides, and about the affected biochemical pathways.
2 Biological resources were available to gain an understanding of the mechanisms of resistance, and provide genes for genetic manipulation, including:
   - resistant bacteria via the laboratory or from the wild;
   - tolerant plants selected in tissue culture;
   - field-selected resistant crops and weeds.
3 Single-gene mechanisms to obtain tolerance were relatively simple to devise. (This is not the case with some of the traits discussed in subsequent chapters.)

Herbicide-tolerant crops have been developed and commercialized by agrochemical companies.

The major commercial impetus for their development flowed from the advantage to the agrochemical/seed industries of producing crops tolerant to specific herbicides, particularly those manufactured/owned by the same company. The engineering of a crop to be tolerant to a particular herbicide allows it to be treated at the optimal times for the reduction of weeds, hence greatly extending the applications and effectiveness of that herbicide. The role of the agrochemical industry in driving forward this technology (and GM crop science in general) will be returned to in this and subsequent chapters.

The use of herbicides in modern agriculture

Weeds, pests, and diseases have a major impact on global crop yield.

Weeds have a significant effect on the yield and quality of crops, as a result of competition for light and nutrients, contamination of the harvested crop and because weed populations harbour pests and diseases. Thus, weeds are one of the three classes of biotic stress that have a major impact on the proportion of world crop yield available for human consumption. Figure 5.1 indicates that the reduction in world crops caused by weeds, pests, and diseases amounts to over one-third of the potential yield, with a roughly equal contribution made by each. Modern agriculture has developed a range of effective herbicides (weedkillers) to tackle the effects of weeds on crop yield. Some of the most useful of these are broad-spectrum herbicides because they are active against a wide range of weeds. However, these can only be used at times when the crop is not itself vulnerable to herbicide action. The development of herbicide-tolerant crops therefore offers the opportunity to spray crops at the most effective time to kill weed species without damaging the crop plants. However, it should be noted that some crop plants are naturally resistant to certain herbicides, and that tolerant strains may appear through the normal processes of mutation and natural

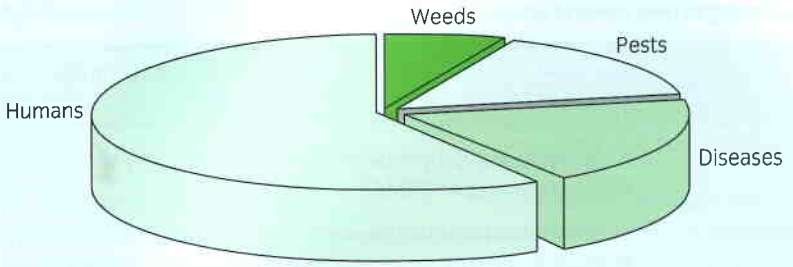


Figure 5.1 The relative proportions of total world crop production lost to weeds, pests, and diseases. The figure shows that of the total potential yield, only 63% is available for human consumption. The remainder is lost in more or less equal proportions to weeds, pests, and diseases.

selection. Thus, the concept of herbicide-tolerant crops is neither novel nor unique to GM technology.

What types of compounds are herbicides?

- Herbicides are inhibitors of plant-specific processes.

By definition, herbicides are much more toxic to plants than to animals. Therefore, it is not surprising that they generally affect plant-specific biological processes. Consider for a moment the type of biochemical pathways that are likely to occur in plants but not in animals. Although one's first thought is likely to be of photosynthesis, many other processes are potential targets. Remember that plants are autotrophic; that is, they can synthesize all their macromolecular components *de novo*. For example, the compounds that are essential in the human diet (vitamins, essential amino acids, etc.) are synthesized by plants, and these biosynthetic pathways are therefore possible targets for herbicides. Many of these pathways are located in the chloroplast (Box 5.1),

BOX 5.1 *Plastids as major biosynthetic factories of plant cells*

- Many plant-specific pathways are located in the plastids.

The plastids are major contributors to the biosynthetic activity in plant cells. The chloroplast is the sole site of the photoreduction of carbon dioxide to phosphoglyceric acid, the photooxidation of water, and photophosphorylation. In addition, all an animal's essential dietary amino acids are synthesized in chloroplasts. The vast majority of lipid biosynthesis occurs in this organelle, as does the synthesis of purines and pyrimidines. The plastids also play a major role in the assimilation of nitrogen, reducing nitrite (produced by reduction of nitrate in the cytosol) to ammonia, and in the assimilation of sulphur, reducing sulphate to sulphide. The chlorophyll pigments of the chloroplast are also produced *in situ*, as are the carotenoid pigments of chromoplasts. Starch biosynthesis is also an important pathway in chloroplasts and is the predominant function of amyloplasts.

The chloroplast is also responsible for a proportion of plant cell protein synthesis. Note that the chloroplast genome encodes all the components required for the plastid translational machinery, as well as for a number of the chloroplast proteins (see Chapter 1).

Table 5.2 Classification of herbicides according to their mode of action

HRAC group	Mode of action	Chemical family	Example
A	Inhibition of ACCase	Aryloxyphenoxy-propionates ('FOPs'), cyclohexanediones ('DIMs')	
B	Inhibition of acetolactate synthase (ALS)	Sulphonylureas, imidazolinones, triazolopyrimidines, pyrimidinyl(thio)benzoate, sulphonylaminocarbonyl-triazolinones	Chlorsulphuron, imazapyr
C1	Inhibition of photosynthesis at photosystem II	Triazines, triazinones, triazolinone, uracils, pyridazinones, phenylcarbamates,	Atrazine
C2	Inhibition of photosynthesis at photosystem II	Ureas, amides	
C3	Inhibition of photosynthesis at photosystem II	Nitriles, benzothiadiazinone, phenylpyridazines	Bromoxynil
D	Photosystem I electron diversion	Bipyridyliums	Paraquat
E	Inhibition of protoporphyrinogen oxidase (PPO)	Diphenylethers, phenylpyrazoles, *N*-phenylphthalimides, thiadiazoles, oxadiazoles, triazolinones, oxazolidinediones, pyrimidindiones, others	
F	Bleaching: inhibition of carotenoid biosynthesis at the phytoene desaturase step	Pyridazinones, pyridinecarboxamides, others	
F2	Bleaching: inhibition of 4-hydroxyphenylpyruvate dioxygenase (4-HPPD)	Triketones, isoxazoles, pyrazoles, others	
F3	Bleaching: inhibition of carotenoid biosynthesis (unknown target)	Triazoles, isoxazolidinones, ureas, diphenylethers	
G	Inhibition of EPSPS	Glycines	Glyphosate
H	Inhibition of glutamine synthetase	Phosphinic acids	Glufosinate ammonium, bialaphos
I	Inhibition of dihydropteroate synthase	Carbamates	Asulam
K1	Microtubule assembly inhibition	Dinitroanilines, phosphoroamidates, pyridines, benzamides, benzenedicarboxylic acids	Oryzalin
K2	Inhibition of mitosis/microtubule organization	Carbamates	
K3	Inhibition of cell division	Chloroacetamides, acetamides, oxyacetamides, tetrazolinones, others	
L	Inhibition of cell-wall (cellulose) synthesis	Nitriles, benzamides, triazolocarboxamides	

Table 5.2 Continued

HRAC group	Mode of action	Chemical family	Example
M	Uncoupling (membrane disruption)	Dinitrophenols	
N	Inhibition of lipid synthesis—not ACCase inhibition	Thiocarbamates, phosphorodithioates, benzofuranes, chlorocarbonic acids	Trichloroacetic acid
O	Action like indole-3-acetic acid (synthetic auxins)	Phenoxycarboxylic acids, benzoic acids, pyridine carboxylic acids, quinoline carboxylic acids, others	2,4-D, Dicamba, Picloram
P	Inhibition of auxin transport	Phthalamates, semicarbazones	
Z	Unknown	Arylaminopropionic acids, pyrazolium, organoarsenicals	

ACCase, acetyl-CoA carboxylase; 2,4-D, 2,4-dichlorophenoxyacetic acid; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; HRAC, Herbicide Resistance Action Committee.
(Data from the Herbicide Resistance Action Committee and the Weed Science Society of America.)

and this has implications for the targeting of many of the transgenic proteins designed to enhance herbicide tolerance.

The herbicidal activity of many herbicides has been found to result from the specific inhibition of a single enzyme/protein. Table 5.2 shows a classification of the major chemical groups of herbicides according to their modes of action. It can be seen that herbicides belong to a wide range of different chemical families, with about 15 broad classes of mode of activity. It is also worth noting that most herbicides only have one mode of action, and that certain enzymes seem to be relatively vulnerable to herbicide activity. For example, five chemical families of herbicide target the enzyme acetolactate synthase (ALS; or more correctly, acetohydroxy-acid synthase or AHAS). ALS catalyses the first reaction in the biosynthetic pathways of branched-chain amino acids (see Figure 5.6, below), and Table 5.3 shows two common classes of herbicide with widely differing chemical structures (sulphonylureas and imidazolinones) that target this enzyme. As will be seen, knowledge of the herbicide's site of action is essential for some strategies aimed at engineering tolerance, but less important for others.

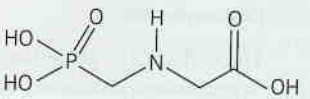
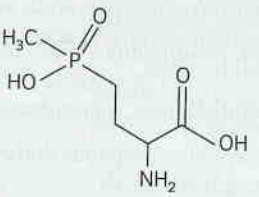
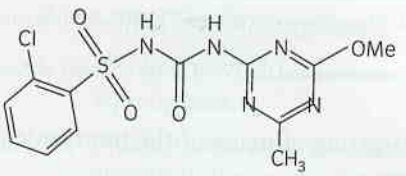
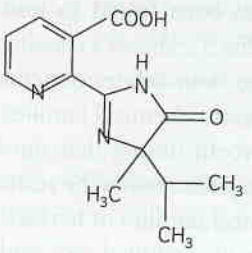
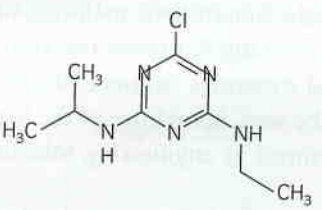
Herbicides differ considerably in structure, properties and mode of action.

The wide range of chemical families shown in Table 5.2 means that herbicides differ greatly in other properties besides their mode of action. For example, they can also be classified according to their:

- site of uptake into the plant (root or shoot);
- degree of translocation within the plant (systemic or contact);
- time of application (preplanting, pre-emergence, postemergence, or preharvesting).

The widely varied chemical properties of these compounds also means they will differ greatly with regard to toxicity, environmental persistence, and biodegradability. The environmental impact of herbicide-tolerant crops will be considered at the end

Table 5.3 The structures of common herbicides affecting biochemical targets

Class of herbicide	Compound/ herbicide	Chemical formula	Inhibited pathway	Target protein
Glycine	Glyphosate (Roundup)		Aromatic amino acids	EPSPS
Phosphinic acid	Phosphinothricin (Basta)		Nitrogen assimilation	Glutamine synthase
Sulphonylurea	Chlorsulphuron (Glean)		Branched-chain amino acids	Acetolactate synthase
Imidazolinone	Imazathapyr		Branched-chain amino acids	Acetolactate synthase
S-Triazine	Atrazine		Photosynthesis	33 kDa protein

EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase.

of this chapter. However, this is a useful place to make the point that an assessment of the risks and benefits of growing a particular herbicide-tolerant crop must take account of the properties of that particular herbicide.

Table 5.3 shows the chemical structure of a few common herbicides that affect well-characterized biochemical targets. This again emphasizes the point that herbicides are a heterogeneous group of compounds with differing modes of action. In

consequence, most transgenic strategies for tolerance to a particular herbicide have to be designed specifically for that class of herbicide. Rather than describe in detail all the herbicides against which tolerance has been engineered, this chapter will adopt a case-study approach to highlight general approaches and strategies.

Strategies for engineering herbicide tolerance

There are four distinct strategies for engineering herbicide tolerance.

The engineering of herbicide tolerance demonstrates the way in which quite different strategies can be used to achieve the same objective. Mullineaux (1992) identifies four distinct strategies for engineering herbicide tolerance, detailed below.

1 *Overexpression of the target protein*. This strategy effectively involves titrating the herbicide out by overproduction of the target protein. For example, if the herbicide is a specific inhibitor of one particular enzyme, production of sufficient excess enzyme will partially overcome the inhibition. Overexpression can be achieved by the integration of multiple copies of the gene and/or the use of a strong promoter plus translational enhancer to drive expression of the gene.

2 *Mutation of the target protein*. The logic behind this approach is to find a modified target protein that substitutes functionally for the native protein and which is resistant to inhibition by the herbicide, and to incorporate the resistant target protein gene into the plant genome. Several sources of resistant proteins can be exploited. Note that both the overexpression and mutated target protein strategies require knowledge of the mode of action of the herbicide.

3 *Detoxification of the herbicide, using a single gene from a foreign source*. Detoxification is a means of converting the herbicide to a less toxic form and/or removing it from the system. This strategy can be contrasted with the previous two because it does not require a detailed knowledge of the site of action. Table 5.4 shows several examples of specific detoxification reactions for common herbicides.

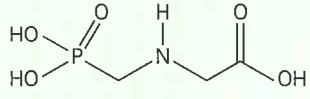
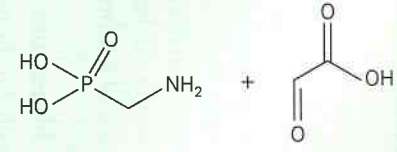
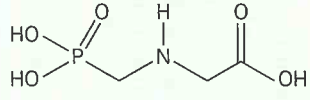
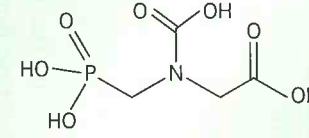
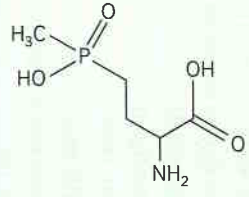
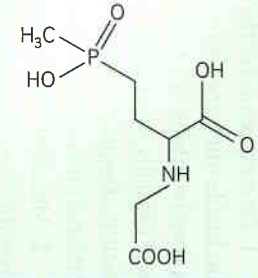
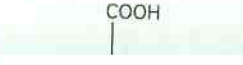
4 *Enhanced plant detoxification*. The aim here is to improve the natural plant defences against toxic compounds. This requires detailed information about endogenous plant detoxification pathways and the mechanisms by which compounds are recognized and targeted for detoxification by the plant.

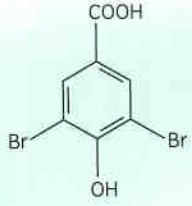
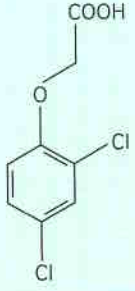
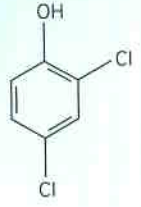
CASE STUDY 5.1 Glyphosate tolerance

Glyphosate is a competitive inhibitor of the enzyme EPSPS.

Glyphosate is a broad-spectrum herbicide that is reputedly effective against 76 of the world's worst 78 weeds, and is marketed as Roundup by the North American chemical company Monsanto. It is a simple glycine derivative (see Table 5.2) that acts as a competitive inhibitor of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). Figure 5.2 shows the similarity in structure between glyphosate and one of the substrates of the enzyme, phosphoenolpyruvate (PEP). Glyphosate binds more tightly to the EPSPS–shikimate 3-phosphate complex than does PEP: its dissociation rate from the complex is 2300 times slower than PEP. Consequently, EPSPS is effectively inactivated once glyphosate binds to the enzyme–substrate complex. EPSPS is a key enzyme in the biosynthetic pathways of the aromatic amino acids phenylalanine, tyrosine, and

Table 5.4 Herbicide detoxification reactions

Compound/herbicide		Enzyme	Metabolite	Source organism
	Glyphosate (Roundup)	Oxidoreductase		*Ochrabactrum anthropi*
	Glyphosate (Roundup)	N-Acetyltransferase		*Bacillus licheniformis*
	Phosphinothricin (Basta)	Acetyltransferase		*Streptomyces hygroscopicus*
	Bromoxynil (Buctril)	Nitrilase		*Klebsiella ozaenae*

	Bromoxynil (Buctril)	Nitrilase		*Klebsiella ozaenae*
	2,4-D	Monooxygenase		*Alcaligenes eutrophus*

2,4-D, 2,4-dichlorophenoxyacetic acid.

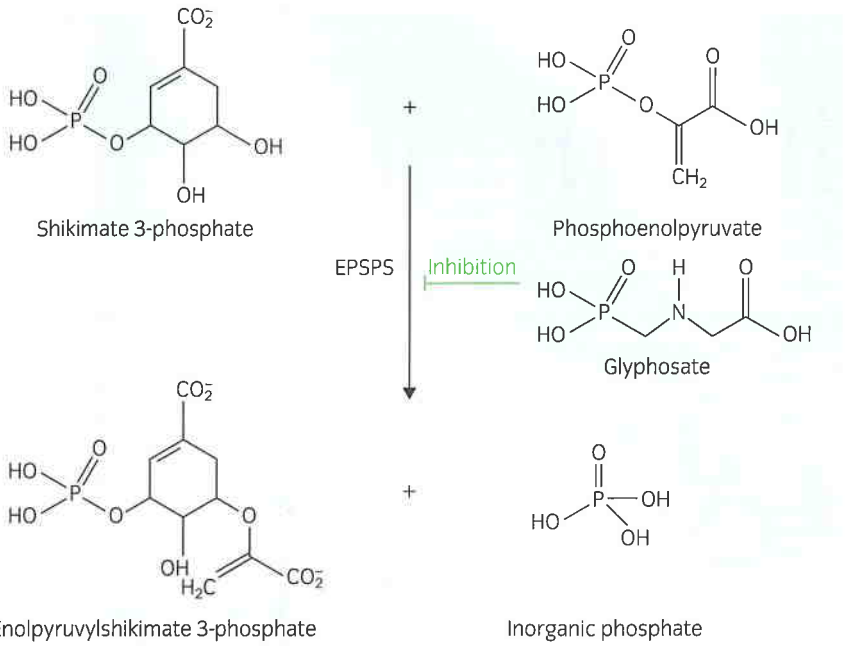


Figure 5.2 **Glyphosate and the reaction catalysed by EPSPS. The biochemical reaction catalysed by EPSPS involves the addition of phosphoenolpyruvate to shikimate 3-phosphate. The structural similarity between glyphosate and phosphoenolpyruvate explains the competitive inhibition of EPSPS by the herbicide.**

tryptophan (Box 5.2). Thus, the herbicidal activity of glyphosate results from its inhibition of the biosynthesis of aromatic amino acids and other products of the shikimate pathway.

From this knowledge of the mode of action of glyphosate, is it possible to predict what effects the herbicide is likely to have on the growth of a treated plant? The first deduction is that protein synthesis will be blocked due to the insufficient supply of aromatic amino acids. The most immediate effects of this inhibition would be expected in regions of the plant involved in rapid growth and division, including the meristems. Certain specialized organs, such as the developing

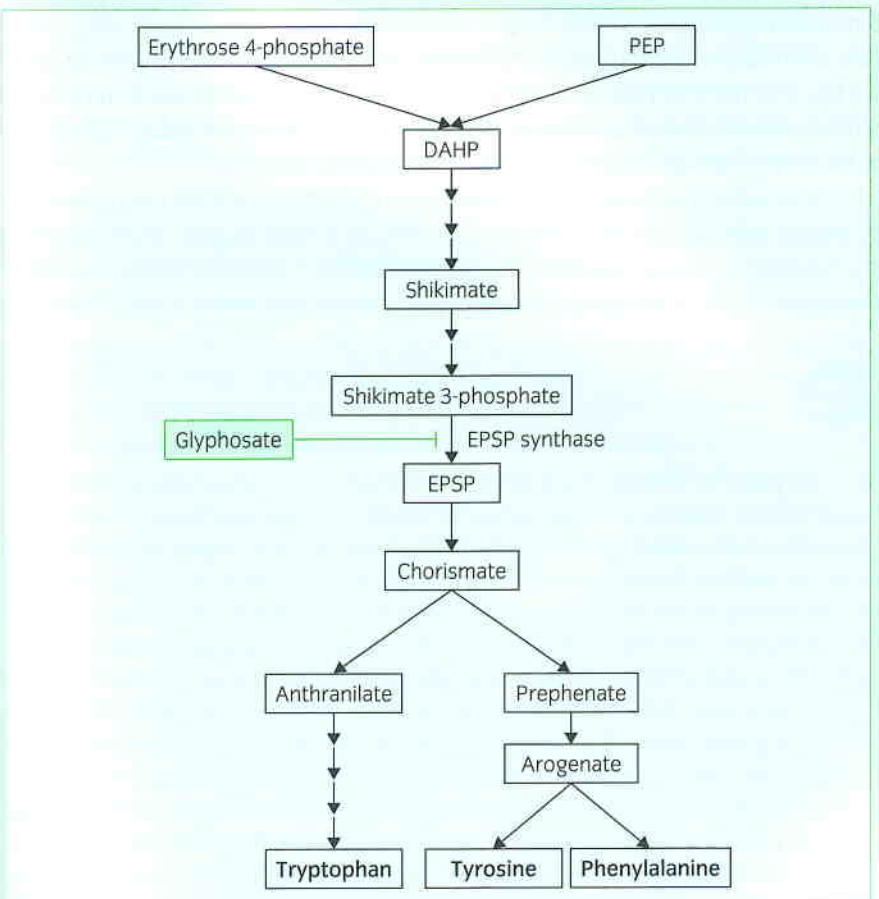
BOX 5.2 *Aromatic amino acid biosynthesis pathways*

The biosynthesis of the aromatic amino acids and related compounds shares a common biochemical pathway up to the formation of chorismate. The pathway to chorismate starts with the condensation of phosphoenolpyruvate (PEP) (from glycolysis) and erythrose 4-phosphate (from the pentose phosphate pathway) to form 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP). The next step involves a complex redox/cyclization reaction producing 3-dehydroquinate. The next two reactions (to form 3-dehydro-shikimate and then shikimate) are catalysed by a bifunctional enzyme in plants. Shikimate kinase then phosphorylates shikimate to produce shikimate 3-phosphate, one of the substrates of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). EPSPS adds the enolpyruvyl side chain to shikimate 3-phosphate to form 5-enolpyruvylshikimate 3-phosphate (EPSP). Finally, chorismate is formed by the elimination of phosphate from EPSP.

BOX 5.2 *Continued*

Chorismate is the precursor of the phenolic and indole rings of the aromatic amino acids, and of other aromatic compounds. Chorismate mutase is the committing enzyme for phenylalanine and tyrosine synthesis, forming prephenate. Prephenate aminotransferase utilizes glutamate or aspartate as amino donors to form arogenate, the immediate precursor of phenylalanine (via arogenate dehydratase) and tyrosine (via arogenate dehydrogenase). These amino acids are themselves the precursors of a wide range of secondary products, including lignins, flavonoids, hydroxycinnamic acids, and alkaloids.

Alternatively, chorismate may be converted to anthranilate by anthranilate synthase, an enzyme complex with two catalytic subunits: the α-subunit catalyses the amination of chorismate and the removal of the enolpyruvyl side chain, while the β-subunit has glutamine aminotransferase activity. Four subsequent steps are involved in the biosynthesis of tryptophan. Tryptophan itself is the precursor of several important secondary products, including IAA, indole alkaloids, indole glucosinolates, phytoalexins, and acridone alkaloids.



Biosynthetic pathways of the aromatic amino acids tryptophan, tyrosine, and phenylalanine.

endosperm, also vigorously accumulate proteins. In addition, other pathways will be affected by the depletion of aromatic amino acids. The shikimate pathway supplies aromatic precursors for a range of phenolic compounds, including lignins, alkaloids, and flavonoids (Box 5.2). Indeed, 20% of the carbon fixed by plants flows through this pathway, primarily for lignin biosynthesis. Indole compounds other than tryptophan are produced by the same pathway, so biosynthesis of auxin (indole-3-acetic acid, IAA) will also be affected by this herbicide. It is therefore not surprising that glyphosate has such a profound effect on plants.

Strategy 1 for glyphosate tolerance: overexpression of a plant EPSPS gene

Three different strategies for the production of glyphosate-tolerant crops have been tested.

Of the four strategies for engineering herbicide tolerance described above, three have actually been tested in the laboratory, two of which form the basis of the current commercial plantings of glyphosate-tolerant crops. It is therefore instructive to compare the three strategies. One of the earliest approaches to engineering glyphosate tolerance involved the overexpression of a plant EPSPS gene. This was facilitated by the isolation of petunia complementary DNA (cDNA) from glyphosate-tolerant tissue cultures. The stepwise selection of petunia cells capable of growing in the presence of increasing amounts of glyphosate led to the isolation of cultures in which the levels of EPSPS enzyme were much higher than normal. This was found not to be due to increased expression of the EPSPS gene, but rather the result of gene amplification, such that there were multiple (up to 20) copies of the EPSPS gene in an otherwise normal petunia genome (see Box 5.3). The EPSPS enzyme was not itself mutated: the tolerance was simply due to the increased amount of enzyme. However, the high levels of EPSPS mRNA made it simpler to isolate the cDNA for this gene and use it for re-introduction into plants.

The observation that the endogenous mechanism of resistance that had been selected for in the petunia cells was one of excess normal EPSPS (as a result of gene amplification) indicates that Strategy 1—overexpression of the target protein—should be feasible. The effect of overexpressing the EPSPS gene in the transgenic petunia was tested in one of the earliest

BOX 5.3 *Gene amplification*

In prokaryotes, the isolation of a strain tolerant to a particular toxic compound would usually result from the selective advantage derived from mutations in the gene coding for the affected protein itself, or in related genes involved in transport, detoxification, or gene regulation. In contrast, the selection of eukaryotic cells in culture resistant to a particular toxic compound often results from the amplification of the gene encoding the target enzyme, rather than from mutations in that gene. This process is particularly observed during a stepwise selection procedure, in which the level of toxin is increased gradually in each round of selection. One of the best-characterized examples is that of methotrexate resistance in cultured mammalian cells. The anticancer drug methotrexate is an inhibitor of the enzyme dihydrofolate reductase (DHFR), which supplies single carbon units for, among other things, thymidine synthesis. It is therefore particularly important in cycling cells for the replication of DNA. A stepwise selection of methotrexate-resistant cells led to the predominance of cells in which the DHFR gene had been specifically amplified in tandem 40–400-fold. This indicates that gene duplication occurs more frequently than mutation in eukaryotes. The stepwise selection applies a quantitative selection pressure for repeated gene duplications, resulting in a process of accelerated evolution.

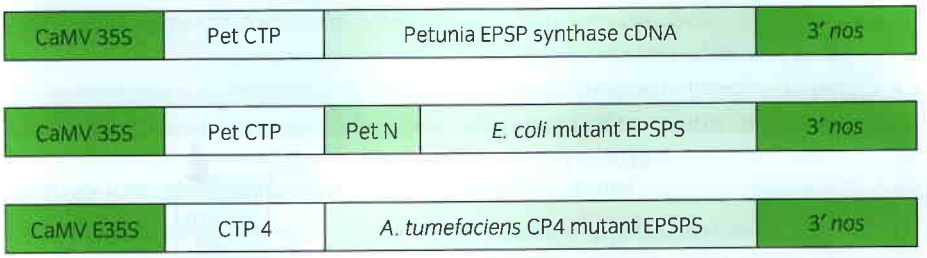


Figure 5.3 Constructs for engineering glyphosate tolerance. Maps of some of the gene constructs that have been used to engineer glyphosate tolerance are shown. The upper two constructs are early experimental designs that were used to demonstrate the feasibility of engineering herbicide tolerance. The first construct directed overexpression of a wild-type petunia (Pet) EPSPS gene, whereas the second shows a resistant *Escherichia coli* gene fused to the N-terminal region of the petunia gene, including the chloroplast transit peptide (CTP). The third construct is one currently used to generate Roundup Ready crops such as soybean and cotton using a resistant EPSPS gene from *A. tumefaciens* with an enhanced CaMV 35S promoter (E35S).

experiments on the engineering of herbicide tolerance. The EPSPS cDNA was fused to the cauliflower mosaic virus 35S promoter and a *nos* terminator sequence in the vector pMON 546 (Figure 5.3) and transformed into petunia using *Agrobacterium*. The use of the plant gene enabled the researchers to avoid one of the major obstacles to transgene expression: protein targeting. As noted above, many of the potential target pathways of herbicides are located in the plastids. The targeting of proteins to the plastid is discussed in Box 5.4. Note that no further

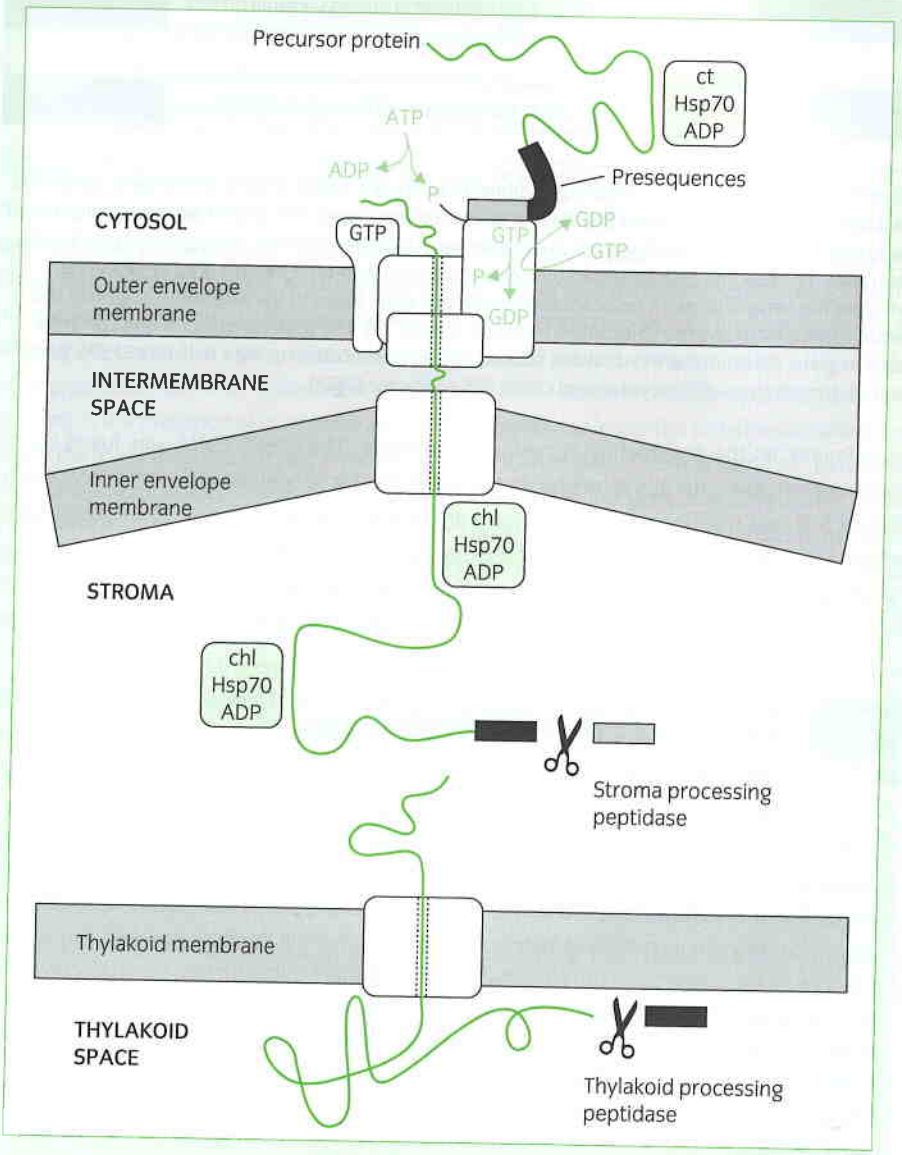
BOX 5.4 *Chloroplast transit peptides and protein targeting*

Some chloroplast proteins are encoded by the plastid genome and translated on 70S ribosomes in the organelle. However, most chloroplast proteins are encoded in the nuclear genome and translated on 80S ribosomes in the cytoplasm. There must therefore be mechanisms for the recognition and transport of proteins destined for the chloroplast. Note that the chloroplast envelope comprises an outer and inner membrane, and that the thylakoids comprise an additional internal membrane system. Thus, there are three distinct compartments in the chloroplast: the intermembrane space of the envelope, the stroma, and the thylakoid lumen. Depending upon the precise destination, a chloroplast protein might be transported across three membrane systems. The means by which all plastid proteins are transported into the chloroplast is by recognition of a sequence of about 40–50 amino acids at the N-terminal end of the protein (the transit peptide). This peptide directs translocation into the stroma, where a specific peptidase removes the transit peptide. Note that the process is post-translational; that is, the protein is transported after synthesis, unlike the co-translational transfer of membrane-bound and secreted proteins into the endoplasmic reticulum during synthesis on rough endoplasmic reticulum-bound ribosomes. The transport process into the stroma appears to involve a complex protein-import apparatus that spans the inner and outer membranes.

Targeting into the thylakoids requires a bipartite transit peptide. Removal of the stromal targeting sequence exposes a second transit peptide that acts as a lumenal targeting peptide.

(Continued overleaf)

BOX 5.4 *Continued*



Protein transport into the chloroplast. chl, chloroplastic; ct, cytoplastic; Hsp70, 70 kDa heat-shock protein. (Adapted with permission from Heldt (1997).)

This directs the protein across the thylakoid membrane into the thylakoid lumen, where it is also removed by a specific protease.

Transit peptides will direct the transfer of a chimaeric non-plastid protein into the chloroplast. Hybrid transgenes containing an N-terminal transit peptide can therefore be constructed so as to target the protein to a specific chloroplast compartment.

manipulation of the cDNA was required to target this protein to the plastid site of activity, since the plant EPSPS cDNA sequence contains its own transit peptide. The result of this experiment was a 40-fold increase in EPSPS activity in the transgenic plants and a tolerance to glyphosate sprayed in the field at a dose two to four times higher than that required to kill wild-type plants.

Strategy 2 for glyphosate tolerance: mutant EPSPS genes

Mutated EPSPS genes have been isolated from a number of glyphosate-resistant bacteria. It is instructive to compare two of the early experiments using glyphosate-resistant genes from bacteria. In one, a mutated *aroA* gene from *Salmonella typhimurium* was inserted between the promoter and terminator sequences of the *ocs* gene of the *Agrobacterium tumefaciens* Ti plasmid. Only a moderate increase in herbicide tolerance was obtained. With reference to Box 5.4, you should be able to explain why tolerance was limited: the prokaryote gene did not have a plastid transit peptide sequence, so the resistant enzyme was not transported into the chloroplast.

The requirement for a functional plastid transit peptide was demonstrated by the construction of a hybrid EPSPS gene by fusion of the C-terminal end of a mutated *aroA* gene from *E. coli* to the N-terminal end of the petunia EPSPS cDNA sequence containing the transit peptide sequence (Figure 5.3). Expression of the chimaeric enzyme increased glyphosate tolerance in transgenic tobacco from 0.01 to 1.2 mmol l^{-1} glyphosate. This experiment was an important validation of the strategy, and demonstrated the feasibility of expressing prokaryotic genes incorporated into the plant nuclear genome, given appropriate promoter and termination signals. However, note that the expression of prokaryotic genes is often not optimal in transgenic plants and extensive modifications may be required to obtain high levels of expression (see Chapter 4).

These early experiments provided useful evidence for the feasibility of Strategy 2 (using resistant target proteins), but also revealed problems associated with it. One is that a mutant enzyme with reduced affinity for a competitive inhibitor may also have a lower affinity (increased K_m, the Michaelis–Menten constant) for the substrate. This proved to be the case with the *E. coli* and *S. typhimurium* genes. For this reason, glyphosate-resistant genes from other sources have been tested for their effectiveness in different plants. A gene from the herbicide-resistant *A. tumefaciens* strain CP4 encodes an EPSPS that is resistant to glyphosate, but retains a low K_m for PEP. This gene, in conjunction with an enhanced CaMV 35S promoter (see Chapter 4, Figure 4.2) and a chloroplast transit peptide sequence from *Arabidopsis* or petunia (Figure 5.3), is incorporated into the current range of Monsanto's major dicotyledonous Roundup Ready crops (soybean, cotton, and oilseed rape). On the other hand, Roundup Ready maize contains a construct optimized for monocotyledonous crops, with a resistant EPSPS gene from maize (isolated after mutagenesis and selection in tissue culture), fused to a rice promoter and maize chloroplast transit peptide sequence.

Strategy 3 for glyphosate tolerance: detoxification by heterologous genes

Alternative strategies have been developed for engineering glyphosate tolerance based upon a specific detoxification mechanism. In soil microorganisms, glyphosate can be degraded by cleavage of the C–N bond, catalysed by an oxidoreductase, to form aminomethylphosphonic acid (AMPA) and glyoxylate (Table 5.4). A gene encoding the enzyme glyphosate oxidase (GOX) has been isolated from a soil organism, *Ochrobactrum anthropi* strain LBAA, and modified by addition of a transit peptide. Transgenic crops such as oilseed rape transformed with this gene show very good glyphosate tolerance in the field. However, this strategy is not generally used

in isolation. Monsanto now employ a dual strategy for canola (a variety of oilseed rape grown in the USA), in which both the resistant *Agrobacterium* CP4 EPSPS gene and the GOX gene are expressed. In addition to enhanced glyphosate tolerance, this approach avoids the accumulation of the herbicide in the tolerant plant, because the glyphosate is broken down into relatively harmless products (glyoxylate is a normal plant metabolite and AMPA can be converted to glycine). This highlights one significant difference between Strategy 2 and Strategy 3. Strategy 2 enables the plant to function in the presence of the herbicide and therefore the herbicide may

BOX 5.5 *Directed evolution of glyphosate acetyltransferase*

Glyphosate is detoxified by acetylation because *N*-acetylglyphosate is not an effective inhibitor of EPSPS and is consequently not herbicidal. The research team associated with Pioneer Hi-Bred (a subsidiary of DuPont) tested a variety of microbial N-acetytransferases for their ability to modify glyphosate, but none was effective. They therefore screened a microbial collection for glyphosate-acetylating activity, by incubating permeabilized cells with acetyl-CoA and glyphosate, and conducting a sensitive assay for *N*-acetylglyphosate. *Bacillus licheniformis* showed the most promising activity, and a glyphosate acetyltransferase (GAT) gene was isolated from two different strains by shotgun cloning of genomic fragments into *E. coli* and assaying for GAT activity. The enzyme belongs to the GNAT superfamily of N-acetyltransferases, and a third gene was subsequently isolated from a different strain of *B. licheniformis* by sequence homology. The enzymes encoded by the three genes were characterized in terms of k_{cat} (the rate constant of the catalytic reaction) and the K_M (a measure of enzyme affinity for substrate). The enzymes had a relatively low k_{cat} (1.0–1.7 min^{-1}) and showed high affinity (i.e. a low K_M) for acetyl-CoA (K_M = 1.0–1.2 μM) but a low affinity for glyphosate (K_M = 1.2–1.6 mM). Given this low efficiency, none of the three genes was capable of conferring significant glyphosate resistance in transgenic plants.

To improve the efficiency of enzyme activity (as measured by a high k_{cat}, a low K_M for glyphosate, or an overall high k_{cat}/K_M ratio), a process of molecular evolution or directed evolution was employed. This involved gene shuffling by random fragmentation and reassembly of fragments derived from the three genes. These were cloned into *E. coli* to create libraries of shuffled gene variants, and the permeabilized cells were screened for GAT activity. Enzymes from the most promising clones were assayed, and shuffled genes from the best 3–12 variants were chosen for the next round of DNA shuffling. Even after the third iteration of shuffling, several enzymes showed approximately 1000-fold improvement in k_{cat}/K_M. At this stage it was possible to start selecting the shuffled libraries on glyphosate, but further improvement of enzyme efficiency was restricted to high k_{cat} activity, with K_M reaching a plateau. Therefore, a synthetic library of sequences was created using the best GAT variant from the fourth iteration as a template, and incorporating sequence diversity from related sequences found in *Bacillus subtilis* and *Bacillus cereus*. At the eighth iteration, further sequence diversity was incorporated based on *Listeria inocua* and *Zymomonas mobilis* hypothetical protein sequences. After 11 iterations, the most efficient GAT variant had a high k_{cat} of 416 min^{-1} and a K_M of 0.05 M glyphosate, giving a k_{cat}/K_M of 8320 min^{-1}, representing an approximately 10 000-fold improvement over the original enzymes! Improved *gat* genes have been introduced into *Arabidopsis*, tobacco, and maize, and are currently undergoing trials to evaluate their glyphosate tolerance in the field.

accumulate to higher levels than those normally found in that crop. In contrast, the detoxification strategy should result either in the destruction of the herbicide, or in the accumulation of a conjugate less harmful than the original compound.

More recently, a glyphosate acetylation detoxification system has been developed by molecular evolution (Box 5.5).

Pleiotropic effects of transgenes

- Transgenes may have unforeseen effects.

It should be noted at this stage that the insertion of a transgene into a plant may result in unforeseen and perhaps undesirable effects. Roundup Ready crops have not been without problems of this type. One phenomenon encountered by Roundup Ready soybeans during hot weather has been splitting of the stems. It has been suggested that this occurs due to the 20% higher lignin content of these plants. A look back to the mode of action of glyphosate should indicate why the introduction of an additional EPSPS gene could result in increased lignin biosynthesis (see Box 5.2). It should be remembered that the plant EPSPS enzyme will still be functional under normal growth conditions in the absence of the herbicide, and that the expression of additional enzyme from the transgene may affect the balance of the relevant metabolic pathways. This lesson will be returned to in subsequent chapters, particularly Chapters 10 and 11 dealing with the manipulation of plant metabolic pathways.

CASE STUDY 5.2 **Phosphinothricin**

- Phosphinothricin is a naturally occurring herbicide.

Glyphosate tolerance is one of the most widespread commercial GM traits. The closest rival to glyphosate in terms of the number and acreage of tolerant crops is the herbicide phosphinothricin (PPT) or glufosinate (Table 5.3). Although both are broad-spectrum herbicides, glyphosate is particularly effective against grasses, and PPT is more effective against broadleafed weeds and least effective against perennials and volunteer cereals. (In this chapter, PPT will be used in preference to glufosinate to avoid confusion between glufosinate and glyphosate. Note also that glufosinate is usually applied as the ammonium salt, and is commonly called glufosinate ammonium.) PPT is unusual among herbicides in being derived from a natural product. Bialaphos is a tripeptide of the form PPT–Ala–Ala, produced by certain *Streptomyces* species. It can be applied directly as a herbicide and has been marketed as such under various trade names, for example Herbiace (Meiji Seika). Bialaphos is converted to the active form L-PPT by proteolytic removal of the alanine residues. PPT was marketed by the German company Hoechst, under the trade name Basta. One point to be aware of in tracking the progress of a particular transgenic crop is the dynamic nature of the agrochemical and biotechnology sector: there have been a number of take-overs and mergers resulting in company name changes. Thus, Hoechst has since undergone a series of mergers such that the Basta brand name has been owned successively by AgrEvo, Aventis, and now Bayer CropScience. A different formulation of PPT is also marketed by Bayer under the brand name Liberty, and complements the LibertyLink lines of transgenic PPT-tolerant crops, which are described below.

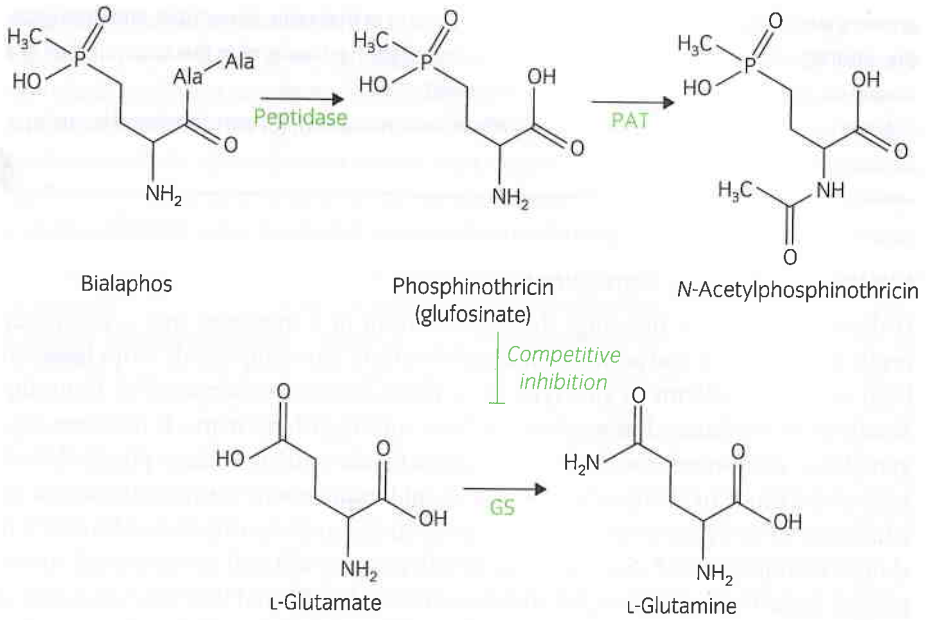


Figure 5.4 The formation, mode of action, and detoxification of PPT. The conversion of bialaphos to PPT involves removal of the two alanine residues by a peptidase. The compound acts as a competitive inhibitor of glutamine synthase (GS), and the figure highlights the structural similarity between PPT and the substrate L-glutamate. The detoxification reaction catalysed by phosphinothricin acetyltransferase (PAT) is also shown.

The herbicidal action of PPT is a result of its competitive inhibition of glutamine synthetase. Figure 5.4 shows the similarity in size and charge between PPT and glutamate, which is one of the substrates of glutamine synthetase. The immediate effect of inhibiting glutamine synthetase is the accumulation of ammonia to toxic levels, which rapidly kills the plant cells. The disruption of glutamine synthesis also inhibits photosynthesis, and it is the combined effects of ammonium toxicity and inhibition of photosynthesis that account for the herbicidal activity of PPT. Uptake of PPT is through the leaf, the speed of which is dependent on many factors, including plant species, stage of growth, air humidity, temperature, and rate of application. Translocation within the plant is limited (unlike glyphosate), and varies according to species. Some limited systemic activity may occur as a result of movement around the leaf, from leaf to leaf, and from leaf to roots. This may be sufficient to suppress the regrowth of perennial weeds that are not killed outright by contact activity. However, it will often not provide the *roots-and-all* kill seen by glyphosate for many perennial grass weeds.

Strategy for PPT tolerance

Basta tolerance is engineered using a detoxification strategy.

The natural occurrence of bialaphos provides a lead to follow in devising a strategy for engineering tolerance. Toxic compounds such as PPT, with a simple structural homology to a common substrate such as glutamate, are likely to be toxic to the host organism. The fact that the compound is synthesized as an inactive precursor is indicative of this fact. It is therefore no

surprising that *Streptomyces* species also contain a detoxification gene that protects the organism from the toxic effects of PPT. The *bar* gene of *Streptomyces hygroscopicus* and the closely related *pat* gene of *Streptomyces viridochromogenes* code for the enzyme phosphinothricin acetyltransferase. The addition of an acetyl group to the amino group of PPT inactivates the compound (Figure 5.4). Thus, transferring this gene to a plant should, in theory, provide resistance against PPT. This approach to the engineering of PPT tolerance was developed by Plant Genetic Systems under contract from Hoechst (Plant Genetic Systems was subsequently acquired by AgrEvo). The *bar* gene has now been integrated into many different plants, usually under the control of the 35S promoter. The current major LibertyLink crop lines are oilseed rape and maize.

The *bar* gene has also proved to be useful as a selectable marker for the transformation and regeneration of transgenic plants. It provides an alternative to selection with antibiotics such as kanamycin, to which different species have a highly varied response (see Chapters 3 and 4). Transgenic plants can be selected directly on PPT medium, but caution is required. Inhibition of glutamine synthetase by PPT causes ammonia accumulation in non-transgenic material and hence death of the plant tissue, but accumulation of ammonia in the non-transformed tissue can also cause problems of toxicity to neighbouring transformed cells.

Prospects for plant detoxification systems

- Plant detoxification mechanisms involve hydroxylation, conjugation, and transport processes.

One other strategy that has yet to be fully exploited is the possibility of enhancing endogenous plant detoxification mechanisms. Many xenobiotic (foreign) compounds are detoxified in plants but the pathways may involve more than one step, such as hydroxylation, conjugation, and transport stages, so it may prove difficult to identify single-gene mechanisms to engineer tolerance. The hydroxylation of compounds involves enzymes such as the cytochrome P450 monooxygenases, which form a large gene family. For example, the analysis of weeds resistant to the herbicide bromoxynil (Table 5.4) revealed that the bromoxynil was being detoxified by an endogenous cytochrome P450 monooxygenase. This offers the opportunity to use endogenous plant genes to enhance resistance against a range of herbicides. The concept has been validated by the demonstration that certain plant cytochrome P450 enzymes confer resistance to phenylurea herbicides. However, more research is required to identify which members of the cytochrome P450 gene family are specific for particular classes of xenobiotics, and which have roles in normal metabolic pathways.

Plant detoxification pathways often involve conjugation to glutathione by glutathione S-transferase (GST) activity, and specific transport of the conjugate into the vacuole. GSTs also comprise a large gene family, some members of which are known to be involved in endogenous metabolic reactions. In some cases, the hydroxylation and conjugation pathways operate in concert. Hence, the resistance of maize to atrazine is ascribed to a two-step pathway involving both 2-hydroxylation and conjugation to glutathione (Figure 5.5). Thus, there is the potential here to enhance endogenous systems, or transfer systems between plant species, once more

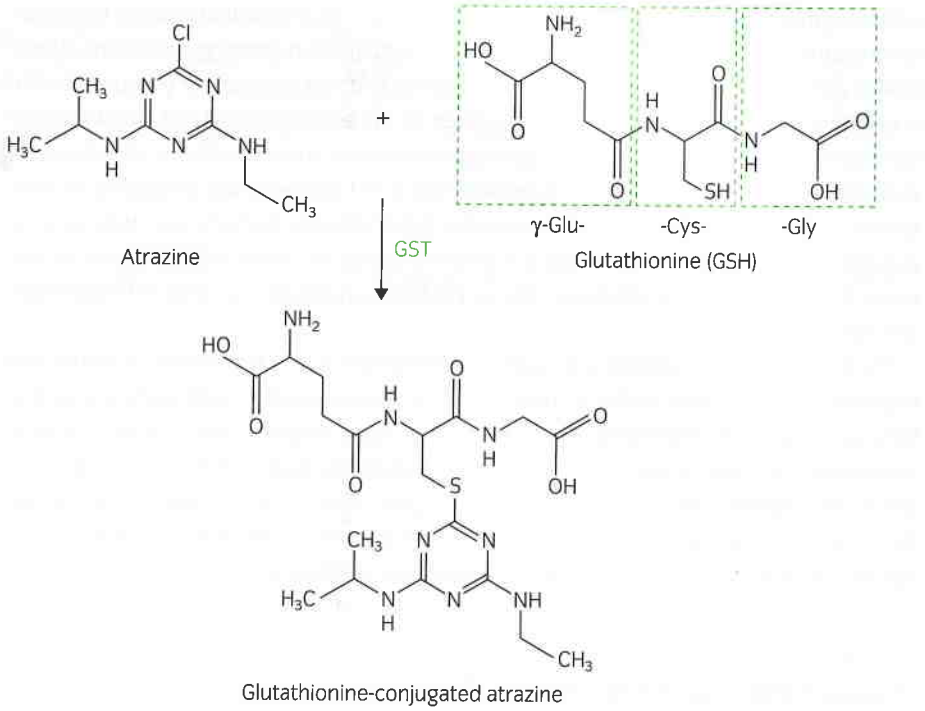


Figure 5.5 The conjugation of atrazine to glutathione. The detoxification of atrazine is a two-stage process involving a 2-hydroxylation step (removing the chlorine residue) prior to the addition of glutathione. Glutathione is a tripeptide in which the key residue is the middle cysteine. The -SH group is involved in a number of redox and conjugation reactions (see also Chapter 9).

information about the functions of these large gene families is available. The exploitation of functional genomics techniques (Chapter 1) will accelerate the acquisition of this knowledge.

Commercialization of herbicide-tolerant plants to date

This chapter has described the two most widespread examples of genetically manipulated herbicide tolerance. Table 5.5 indicates those crops resistant to a number of other herbicides that have been developed to the field-trial stage. It is important to note that some of these have not been widely planted commercially. The atrazine-tolerant crops, for example, have not been developed further, given the environmental concerns about the use of this class of persistent herbicide.

The table gives the strategy employed in each case. All these examples use either the mutant-target-enzyme approach, or the prokaryotic detoxification-gene approach, which have been described in detail when considering the glyphosate and PPT case studies. However, one more example is worth exploring further, because it demonstrates the use of homologous recombination to modify an endogenous plant gene to engineer herbicide tolerance.

Table 5.5 Herbicide resistant crops grown in the field

Class of herbicide

Table 5.5 Herbicide-resistant crops grown in the field

Class of herbicide	Compound/herbicide	Transgene/mechanism	Companies	Crops
Glycine	Glyphosate (Roundup)	*Agrobacterium* CP4-resistant gene	Monsanto	Alfalfa, cotton, oilseed rape, soybean, wheat
			Monsanto/Syngenta	Sugar beet
	Glyphosate (Roundup)	Maize resistant gene	Monsanto	Maize
	Glyphosate (Roundup)	Oxidoreductase detoxification	Monsanto	Maize, oilseed rape, soybean
	Glyphosate (Roundup)	Acetylation detoxification	DuPont-Pioneer Hi-Bred	Soybean, maize
Phosphinic acid	Phosphinothricin (Basta), (Liberty)	*bar* gene; PAT detoxification	Bayer CropScience	Maize, rice, wheat, cotton, oilseed rape, potato
			Syngenta	Tomato, sugar beet
Sulphonylurea	Chlorsulphuron (Glean)	Mutant plant acetolactate synthase	DuPont-Pioneer Hi-Bred	Rape, rice, flax, tomato, sugar beet, maize
Imidazolinone	(Lightening, Beyond)	Mutant plant acetolactate synthase	BASF	Canola, maize, rice, sunflower, wheat (Clearfield non-GM technology)
S-Triazine	Atrazine (Lasso)	Mutant plant chloroplast *psbA* gene	DuPont	Soybean
Nitriles	Bromoxynil (Buctril)	Nitrilase detoxification	Calgene/Bayer CropScience	Cotton, oilseed rape, potato, tomato
Phenoxy-carboxylic acids	2,4-D	Monooxygenase detoxification	Bayer CropScience	Maize, cotton

2,4-D, 2,4-dichlorophenoxyacetic acid; PAT, phosphinothricin acetyltransferase.

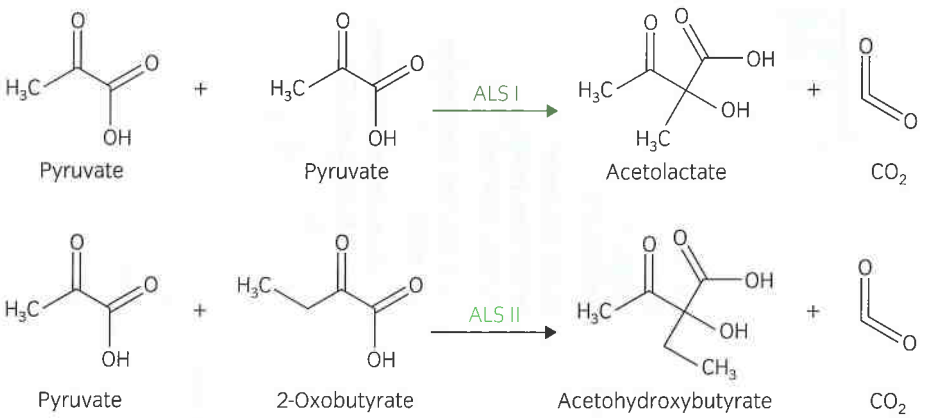


Figure 5.6 The reactions catalysed by ALS forms I and II. The formation of acetolactate by ALS I is the first step in the biosynthesis of leucine and valine, whereas the similar reaction catalysed by ALS II is a key step in isoleucine synthesis.

CASE STUDY 5.3 Engineering imidazolinone tolerance by targeted modification of endogenous plant genes

As stated near the beginning of this chapter, two important classes of herbicide (sulphonylureas and imidazolinones) have the same mode of action; that is, they inhibit the enzyme ALS (see Table 5.3 and Figure 5.6). This blocks the synthesis of the aliphatic amino acids isoleucine and/or leucine and valine. Herbicide-tolerant forms of this enzyme have been isolated from a range of species, from bacteria and yeast to plants, and some of these have been used to engineer herbicide tolerance by standard Strategy 2-type approaches. The sequence analysis of various resistant ALS genes also provided information about the precise changes in amino acid sequence responsible for the resistant character. Researchers at Pioneer Hi-Bred International used this type of information to predict that a single base change in the endogenous maize ALS gene family would be sufficient to change Ser-621 (encoded by AGT) to Asn-621 (AAT) and that this should confer tolerance to the herbicide Lightning (a mixture of imazathapyr (Table 5.3) and imazapyr). This was actually achieved by bombarding maize cells with an oligonucleotide made up of a combination of DNA and RNA bases, with a 32-base section having exact homology to the target sequence of the endogenous maize genes, apart from the single-base mismatch to give the desired mutation. Cells that were able to grow and develop callus on medium containing imazathapyr were selected, and plants were regenerated at an estimated transformation efficiency of 10^{-4}.

● The very low rate of homologous recombination in plants is a barrier to the manipulation of endogenous plant genes.

Some nine independently transformed plants were tested for Lightning tolerance: three were resistant to fourfold the normal field dose, whereas four others were only slightly injured by the fourfold dose and were able to tolerate the normal field dose. The remaining two plants were as susceptible to the herbicide as control plants. There was a direct correlation between Lightning tolerance and the sequence of ALS genes in the transformed plants. The highly resistant plants contained the predicted AGT→AAT mutation, whereas the moderately resistant plants showed

mutations within a few bases of the target site. In contrast, the sequence of the ALS genes was not altered in the susceptible plants.

The conclusion drawn from this work is that although homologous recombination is a rare event in plants, it can be used to change endogenous gene sequences under certain circumstances. Does this therefore mean that many more traits will be engineered by this type of procedure in the future? There are certainly many advantages to the manipulation of an endogenous gene *in situ*, since it avoids many of the problems associated with transgene cloning, modification, expression, and targeting, as well as the requirement for a selectable marker gene, unpredictable effects following random insertion into the genome, etc. However, this procedure will only be suitable for certain types of genetic modification. First, the precise base change needed to produce the desired mutation must be known. Second, the targeted gene mutation must confer a trait that is selectable at this very low frequency of transformation. This is ideal for herbicide tolerance, but generally not for the types of trait such as pest and disease resistance that are discussed in the next three chapters. This point is demonstrated by the fact that the majority of examples of gene targeting have focused on the ALS genes. Thus, chlorsulphuron-resistant ALS genes have been produced in tobacco, changing Pro-196 to Ala, Thr, Glu, or Ser and by a Try-573→Leu substitution. Similarly, in rice, three different chimaeric RNA/DNA oligonucleotides designed to modify Pro-171→Ala, Trp-548→Leu, and Ser-627→Ile conferred chlorsulphuron resistance.

The environmental impact of herbicide-tolerant crops

- The adoption of herbicide-tolerant crops could have positive or negative environmental consequences.

The predominance of herbicide-tolerant crops in terms of their development and commercial growing was highlighted at the beginning of this chapter. Some of the reasons for this trait maintaining its position in league tables of GM crops have been discussed. Scientifically, herbicide tolerance has a number of advantages that facilitated its rapid development. A number of different single-gene strategies using accessible genes are possible, and the trait itself can be used as a selectable marker. These factors, combined with the research and development impetus provided by the agrochemical industry, inevitably led to their prime position. However, it should be noted that herbicide-tolerant crops do not top the league table just for these reasons. They have maintained their leading position because of the rapid adoption of these crops in countries where the technology has been accepted.

In the USA, where the greatest area of GM crops are grown, the proportion of herbicide-tolerant soybean rose from 17% of the total soybean planting in 1997 to 68% in 2001. Similarly, herbicide-tolerant cotton expanded from 10% of cotton in 1997 to 56% in 2001. This rapid adoption of herbicide-tolerant crops begs a number of questions. The first one is whether farmers are adopting these crops so rapidly because there is a clear commercial advantage, and if so, what is it? The second one relates to the environmental impact of this rapid switch in growing patterns, and hence herbicide-usage patterns. Box 5.6 presents one analysis of the economic benefits of growing glyphosate-tolerant soybean, and its impact on herbicide use.

BOX 5.6 *Roundup Ready soybean: glyphosate usage*

It has been calculated that from 1997 to 1998 there was an 81% increase in glyphosate use (up by 5540 t) in line with an increase in the proportion of GM soybean from 13 to 36% of the crop. However, this was more than compensated for by a reduction in the use of other herbicides on soybean of 8990 t, resulting in a net reduction in total herbicide use of 3360 t (–9.7%). Since glyphosate has better properties than some of the other herbicides in terms of toxicity, environmental persistence, and biodegradability, this is claimed to be of net benefit to the environment. This also has an impact on the production costs of the crop. For example, typical 1999 production costs (in US dollars) of Roundup Ready and non-GM soybean have been calculated to be approximately $225 and $250 per hectare, respectively, showing a net saving of $25 per hectare. The large saving in herbicide costs more than compensates for the technology fee charged for the Roundup Ready crop.

It is clear that there is a significant reduction in production costs associated with the use of Roundup Ready soybeans, derived principally from the reduced use of herbicides. In addition, it has been estimated that most of the economic benefits accrue to the farmer, as demonstrated in the table.

Beneficiaries	Estimated benefits (million $US)	Distribution of benefits (%)
Seed companies	32	3
US consumer	42	4
Technology inventor	74	7
US farmer	769	76
Total benefits	1061	100

(Data from Falck-Zepeda *et al.* (2000).)

This indicates that although the use of glyphosate increased compared with that for non-transgenic soybean, the total use of herbicides decreased. Indeed, the major beneficiary of this technology was the farmer rather than the producer, mainly by way of a marked overall reduction in herbicide costs. Thus, the assumption that herbicide-tolerant crops will inevitably lead to a greater use of herbicides needs to be qualified. The use of the target herbicide may well increase, but this may displace or reduce the requirement for other herbicides, such that there is a net reduction in their use. Since the environmental impact of herbicides such as glyphosate and PPT is thought to be lower than many of the compounds they are displacing, the producers of herbicide-tolerant crops can claim that the adoption of their crops will have a positive effect on the environment. However, there are other environmental concerns about the rapid adoption of herbicide-tolerant crops. One area of concern is the reduction of biodiversity as a result of the more efficient removal of weed species from arable land. These concerns relate more widely to current intensive agricultural

practices, and lie outside the direct scope of this book. Another area of concern relates to the possibility of encouraging the development of herbicide-resistant weeds, and is discussed below.

The development of super-weeds

One of the major concerns about the use of herbicide-tolerant crops is that their introduction will lead to the appearance of so-called super-weeds, which could evade control by the commonly used herbicides. It is important to put these fears into context. Previously we have discussed the fact that some crops are already tolerant to certain herbicides, and that herbicide-resistant weeds may appear naturally wherever there is a selective pressure created by the repeated use of the same herbicide. Thus, the problem of herbicide-resistant weeds is not a new phenomenon unique to GM technology and, bearing in mind the large armoury of herbicides listed in Table 5.2, is not one that need pose a major threat. Nevertheless, it is sensible to ask whether the widespread use of herbicide-tolerant crops will make a difference to the number, rate of appearance, and type of resistant weeds because: (1) they encourage the repeated use of the same herbicide, and (2) the herbicide-resistance gene is transferred to a weed population by one of the processes of gene flow described below.

Herbicide-resistant weeds could theoretically arise by three types of mechanism.

● Super-weeds could arise by three different mechanisms.

1 The herbicide-tolerant crop itself appears as a 'volunteer' weed in fields where rotational crops are grown. This volunteer population could reproduce outside of cultivation and form a self-sustaining weed population.

2 Pollen from the herbicide-tolerant crop fertilizes weedy relatives of the crop plant, producing herbicide-tolerant hybrids. Subsequent backcrossing of these hybrids with the weed species could lead to introgression of the herbicide-tolerant trait into the weed population.

3 There is horizontal gene transfer (as opposed to the vertical transmission in points 1 and 2) by other mechanisms (e.g. viruses) that spread the herbicide-tolerant trait into a much wider range of plant species.

Just as the environmental impact of each herbicide should be considered separately, it is also important to treat different crops on a case-by-case basis. Thus, certain crops are normally more prone to form volunteer weeds than others, and the presence of a herbicide-tolerant strain could be a problem if that herbicide is normally used to clear the volunteers.

However, the large arsenal of herbicides shown in Table 5.2 should ensure that volunteer weeds can be cleared from cultivated land for rotational crops. It must be borne in mind that the concept of a weed is the product of a complex mix of characteristics that do not normally include herbicide tolerance. When considering gene flow from a GM crop to other crops and weed species, the nature of each individual crop must also be considered. For example, the spread of pollen from out-crossing crops is more of a problem than with crops that are self-crossing. Another factor to

consider is the proximity of closely related weed species. Thus, maize, soybean, and cotton have no compatible wild-type relatives in the countries where GM varieties are widely grown (USA and Canada). Wheat, oilseed rape, and sugar beet are grown in close proximity to related species, but only the latter two are out-crossing. For this reason, oilseed rape and sugar beet have been used as crops in worst-case analyses for the study of gene flow into weeds. The studies that have been carried out to date tend to indicate that there is detectable gene flow from crops to weed species, but that transgene introgression under field conditions is probably rare. In order to minimize this potential gene flow, it is necessary to predict the distance that pollen can travel from each GM crop, and to establish appropriate buffer distances to prevent the fertilization of weedy relatives. However, many of the studies to determine buffer distances have been on a relatively small scale. A recent large-scale study using herbicide-tolerant oilseed rape (produced by non-transgenic means) does indicate that oilseed rape pollen can travel much further than had been suspected, but that nevertheless, the amount of gene flow to non-resistant plants was minimal.

There is as yet little evidence that horizontal gene flow between plants poses a particular problem for the transfer of transgenes into unrelated species. What is clear about the entire issue of environmental impact of herbicide-tolerant crops is that much more research is required to establish the full extent of all types of gene flow between plants in the environment. Thus, a recent review of the environmental risks and benefits of GM crops concluded that:

1 the risks and benefits of GM crops are not entirely certain or universal;
2 the ability to predict ecological impacts of any introduced species (GM or not) is difficult, and available data have limitations;
3 some benefits and risks may exist that have not yet been identified or addressed in published literature;
4 the quantity and quality of different GM crops that may eventually be developed merit special consideration for risk assessment;
5 better evaluation of potential benefits will help risk managers know how these balance with potential risks;
6 measures developed to prevent gene transfer to wild plants can reduce the potential environmental impacts and prolong potential benefits.

SUMMARY

This chapter has given an overview of the genetic manipulation of herbicide-tolerant plants. The wide range of different chemical families of herbicides and their modes of action have been described. Four basic strategies for the genetic engineering of herbicide tolerance have been discussed with reference to a number of case studies. It has been concluded that Strategy 2 (resistant target protein) and Strategy 3 (specific detoxification) are currently the most effective approaches. However, future developments

may well include the more precise enhancement of endogenous detoxification mechanisms and the targeted mutation of endogenous resistance genes. Concerns about the environmental impact of herbicide-tolerant crops have been touched upon, with a view to returning to these wider issues in Chapter 12.

The next four chapters deal with the genetic manipulation of resistance to other stresses: pests, diseases, and abiotic stress. Some of the concepts introduced in this chapter will be recurring themes in the subsequent chapters. However, we will also introduce new ideas and techniques in a logical sequence as we progress through the next chapters.

FURTHER READING

Visit www.oxfordtextbooks.co.uk/orc/slaterplants2e/ for hyperlinked references that take you directly to online abstracts for the journal articles listed below:

Plant biochemistry

Buchanan, B. B., Gruissem, W., and Jones, R. L. (2002) *Biochemistry and Molecular Biology of Plants*. American Society of Plant Physiologists, Rockville, MD.

Heldt, H.-W. (1997) *Plant Biochemistry and Molecular Biology*. Oxford University Press, Oxford.

Herbicide-tolerant crops

Cole, D. J. (1994) Molecular mechanisms to confer herbicide resistance. In *Molecular Biology in Crop Protection* (G. Marshall and D. Walters, eds), pp. 146–176. Chapman & Hall, London.

Falck-Zepeda, J. B., Traxler, G., and Nelson, R. G. (2000) Rent creation and distribution from biotechnology innovations: the case of *Bt* cotton and herbicide-tolerant soybeans in 1997. *Agribusiness* **16**, 21–32.

Mullineaux, P. M. (1992) Genetically engineered plants for herbicide resistance. In *Plant Genetic Manipulation for Crop Protection* (A. M. R. Gatehouse, V. A. Hilder, and D. Boulter, eds), pp. 75–107. CAB International, Wallingford.

Directed evolution

Castle, L. A., Siehl, D. L., Gorton, R., Patten, P. A., Chen, Y. H., Bertain, S., Cho, H.-J., Duck, N., Wong, J., Liu, D., and Lassner, M. W. (2004) Discovery and directed evolution of a glyphosate tolerance gene. *Science* **304**, 1151–1154.

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Kovchenko, A. and Willmitzer, L. (2003) Chimeric RNA/DNA oligonucleotide-based site-specific modification of the tobacco acetolactate synthase gene. *Plant Physiology* **132**, 174–184.

Zhu, T., Peterson, D. J., Tagliani, L., St Clair, G., Baszczynski, C. L., and Bowen, B. (1999) Targeted manipulation of maize genes *in vivo* using chimeric RNA/DNA oligonucleotides. *Proceedings of the National Academy of Sciences USA* **96**, 8768–8773.

Zhu, T., Mettenburg, K., Peterson, D. J., Tagliani, L., and Baszczynski, C. L. (2000) Engineering herbicide-resistant maize using chimeric RNA/DNA oligonucleotides. *Nature Biotechnology* **18**, 555–558.

Environmental impact

Rieger, M. A., Lamond, M., Preston, C., Powles, S. B., and Roush, R. T. (2002) Pollen-mediated movement of herbicide resistance between commercial canola fields. *Science* **296**, 2386–2388.

Senior, I. J. and Dale, P. J. (2002) Herbicide-tolerant crops in agriculture: oilseed rape as a case study. *Plant Breeding* **121**, 97–107.

Wolfenbarger, L. L. and Phifer, P. R. (2000) The ecological risks and benefits of genetically engineered plants. *Science* **290**, 2088–2093.

Web-links

AgBiotechInfonet, web-link 5.1: www.biotech-info.net/herbicide-tolerance.html

AgBioTechNet, web-link 5.2: www.agbiotechnet.com/

Agricultural Biotechnology Council, web-link 5.3: www.abcinformation.org/benefits.php

Bayer CropScience BioScience, web-link 5.4: www.bcsbioscience.co.uk/

Biotechnology Industry Organisation, web-link 5.5: www.bio.org/foodag/

Council for Biotechnology Information, web-link 5.6: www.whybiotech.com

CropGen, web-link 5.7: www.cropgen.org/

DuPont Biotechnology, web-link 5.8: www2.dupont.com/Biotechnology/en_US/

Felsot, A. *Agrichemical and Environmental News* 2000–2001 [articles in issues 173, 175, 176, and 178], web-link 5.9: www.tricity.wsu.edu/aenews/Feb01AENews/Feb01AENews.htm#anchor5338542

GEO-PIE Genetically Engineered Organisms, Public Issues Education Project, Cornell University, web-link 5.10: www.geo-pie.cornell.edu/gmo.html

Herbicide Resistance Action Committee and the Weed Science Society of America, web-link 5.11: www.plantprotection.org/HRAC/MOA.html

James C. (2001, 2005) *International Service for the Acquisition of Agri-biotech Applications*, web-link 5.12: www.isaaa.org/

Monsanto Biotechnology Knowledge Centre, web-link 5.13: www.biotechknowledge.monsanto.com

Montsanto company, web-link 5.14: www.monsanto.com/monsanto/layout/default.asp

Pesticide Action Network Pesticide Database, web-link 5.15: www.pesticideinfo.org

Pioneer Hi-Bred, web-link 5.16: www.pioneer.com/biotech/default.htm

USDA Economic Research Service, web-link 5.17: www.ers.usda.gov/Briefing/Biotechnology/