

REVIEWS

Engineering herbicide tolerance into crops

Elli Oxtoby and Monica A. Hughes

Herbicide treatments of crops allow economic weed control and provide cost-effective increases in agricultural productivity. Although herbicides currently in use combine a high degree of effectiveness with favourable production costs, non-toxicity and rapid biodegradation, they often lack selectivity, thus limiting their use to pre-emergence applications. Engineering herbicide tolerance into crops is a new way of conferring selectivity and enhancing crop safety and production. This article reviews recent approaches and results in this area of molecular research.

To be useful, herbicides must distinguish between crop plant and weed. This may be problematic; although herbicides are designed to affect processes unique to plants – photosynthesis, for example – these processes are common to both crops and weeds. Consequently, at present, selectivity is based on differential herbicide uptake between weed and crop, or controlled timing and site of application, or detoxification of the herbicide by the crop plant¹. Public concern about the environment increases the demand for herbicides that are rapidly biodegradable, highly effective, and not toxic to animals. For the past decade, commercial strategies and research objectives have reflected this change of attitude: their aim is to reduce overall herbicide use through substitution of more effective, environmentally acceptable, and less costly products. The development of crop plants selectively tolerant to such herbicides provides new means of achieving this.

Genetic variation in herbicide resistance can be found in nature. For example, more than twenty weed species have produced forms resistant to the herbicide triazine² and a few weed species have evolved

paraquat resistance³. Multiple herbicide resistance has also arisen in the wheat weed, *Alopecurus myosuroides*, as a result of selection due to treatment with a single compound. However, the biochemical and genetic basis of this resistance is not fully understood⁴. In addition, mutant-selection techniques have led to the isolation of tobacco⁵, maize⁶ and *Arabidopsis*⁷ plants resistant to sulphonylureas and imidazolinones. Conventional genetics has indicated that, in many cases, resistance is due to a single dominant mutation. The characteristic of herbicide resistance is, therefore, considered to be a good candidate for plant genome modification, since it is potentially valuable and may be determined by a single gene.

Successful first steps towards engineering herbicide-resistant crops fall into two areas: (1) altering the level and sensitivity of the target enzyme for the herbicide; and (2) introducing a gene encoding an enzyme that detoxifies the herbicide (Table 1).

Altering the level and sensitivity of the target of the herbicide

The increasing knowledge of modes of action of herbicides, and rapid progress in molecular genetics have led to the isolation and modification of numerous genes encoding the target proteins for herbicides (from both plants and microorganisms). Commercially important herbicides against which resistance

has been engineered using this approach can be divided into photosynthesis inhibitors and inhibitors of amino acid biosynthesis.

Inhibitors of photosynthesis

- **Triazines** – this group of compounds kills plants by disrupting photosynthetic electron transport in photosystem II. They are known to interfere with binding of plastoquinone to the Q_B protein, a hydrophobic protein that is embedded in the thylakoid membrane of the chloroplasts. It is encoded by *psbA*, a gene located in the chloroplast genome. Triazine-resistant forms of a number of microbial and plant species have arisen spontaneously. In these resistant plants, the Q_B protein produced differs from the wild-type protein by a single amino acid substitution and is, therefore, unable to bind to the herbicide^{8,9}. However, this character is maternally inherited; the inability to introduce foreign genes into chloroplasts reproducibly has been a major problem for engineering triazine-resistant crops¹⁰.

To bypass this technical problem, the *psbA* mutant gene from *Amaranthus hybridus* was converted into a nuclear gene by fusing its coding region to transcription-regulation and transit-peptide-encoding sequences of a nuclear gene. These chimaeric constructs were introduced into the nuclear genome of tobacco using *Agrobacterium* Ti transformation techniques. The protein product of nuclear *psbA* was identified in the chloroplasts of transgenic tobacco plants. These plants showed increased tolerance to atrazine, demonstrating that the transported *psbA* product functions in photosynthesis¹¹.

Additional attempts to transfer atrazine resistance include protoplast fusion between *Solanum*¹² and between *Brassica*¹³ lines. The application of this technology is, however, limited because very few systems are yet available for the regeneration of plants from protoplasts.

Studies in *Brassica* indicate that the introduction of complete chloroplast genomes from a different species may have major consequences for yield. Maternally inherited triazine resistance has been introduced into *Brassica napus* from

E. Oxtoby and M. A. Hughes are at the Department of Biochemistry and Genetics, Medical School, The University, Newcastle-upon-Tyne NE2 4HH, UK.

Table 1

Herbicide, primary target and successful steps towards engineering herbicide-resistant plants

Inhibited pathway	Herbicide	Primary target	Strategy for engineering resistance	
			Target modification	Detoxification
Photosynthesis	Atrazine	Q _B protein	Mutant <i>psbA</i> gene (plant)	GST gene (plant)
	Bromoxynil	Q _B protein	—	<i>bxn</i> gene (bacterial)
Amino acid biosynthesis	Phosphinothricin	Glutamine synthase (GS)	GS gene amplification	<i>bar</i> gene (bacterial)
	Sulfonylurea and imidazolinone	Acetolactate synthase (ALS)	Mutant ALS genes (plant)	—
	Glyphosate	5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS)	EPSPS gene amplification; Mutant <i>AroA</i> gene (bacterial)	—
Growth regulation	2,4-D	Not known	—	<i>tfdA</i> (bacterial)

Brassica campestris via sexual crossing¹⁴. After eight backcross generations to *B. napus* the nuclear genome will be almost isogenic, but by using the resistant plant as the female parent in each cross the *B. campestris* chloroplast genome will be retained. The resulting cultivar has been released commercially, but its yield is reduced by 30%. Thus, in this crop, the combination of *B. campestris* chloroplasts in *B. napus* nuclear background has a significant effect on plant growth. The extent to which this problem can be overcome by introducing a single chloroplast gene rather than the complete genome has yet to be tested. Despite extensive research and the availability of a number of resistance genes, engineering triazine resistance into crops via transformation methods is still problematic.

Inhibitors of amino acid biosynthesis

- **Glyphosate** (N-phosphonomethyl glycine) – this herbicide acts by specifically inhibiting the enzyme 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) (Fig. 1). This is currently the most extensively used non-selective herbicide and is effective in controlling 76 of the world's 78 worst weed species. Glyphosate is not toxic to animals and is rapidly degraded by soil microorganisms. Tolerance to glyphosate has been engineered into various crops by introducing glyphosate-tolerant variant EPSPS enzymes¹⁵ or by introducing genetic constructions for the overproduction of EPSPS¹⁶.

Glyphosate-tolerant transgenic tobacco¹⁵ and, later, tomato¹⁷ plants were generated by transferring the

mutant *AroA* gene (which encodes EPSPS) from *Salmonella typhimurium*. In plants, EPSPS is predominantly localized in the chloroplast; the bacterial *AroA* gene, however, lacks a chloroplast transit-peptide sequence and, consequently, gives rise to a cytoplasmic form of the enzyme. None the less, transgenic plants expressing the bacterial EPSPS in the cytoplasm showed tolerance to glyphosate. The one drawback to these plants was that, after foliar herbicide treatments, the transformants were smaller than the unsprayed controls. It is suggested that the production of a tolerant phenotype in these transgenic plants may be due either to the presence of a cytoplasmic shikimate pathway or to the transport of substrates and products of reactions across the chloroplast membrane¹⁵.

Although targeting the mutant enzyme to the same cell compartment as the endogenous plant enzyme is not always necessary to confer tolerance, it might significantly enhance it. A glyphosate-resistant EPSPS from *E. coli* was successfully targeted to chloroplasts by fusing it 3' to the transit-peptide sequence of the corresponding plant enzyme. Such chimaeric constructs yielded transgenic tobacco plants more tolerant to the herbicide than plants that overexpress the wild-type EPSPS gene¹⁸.

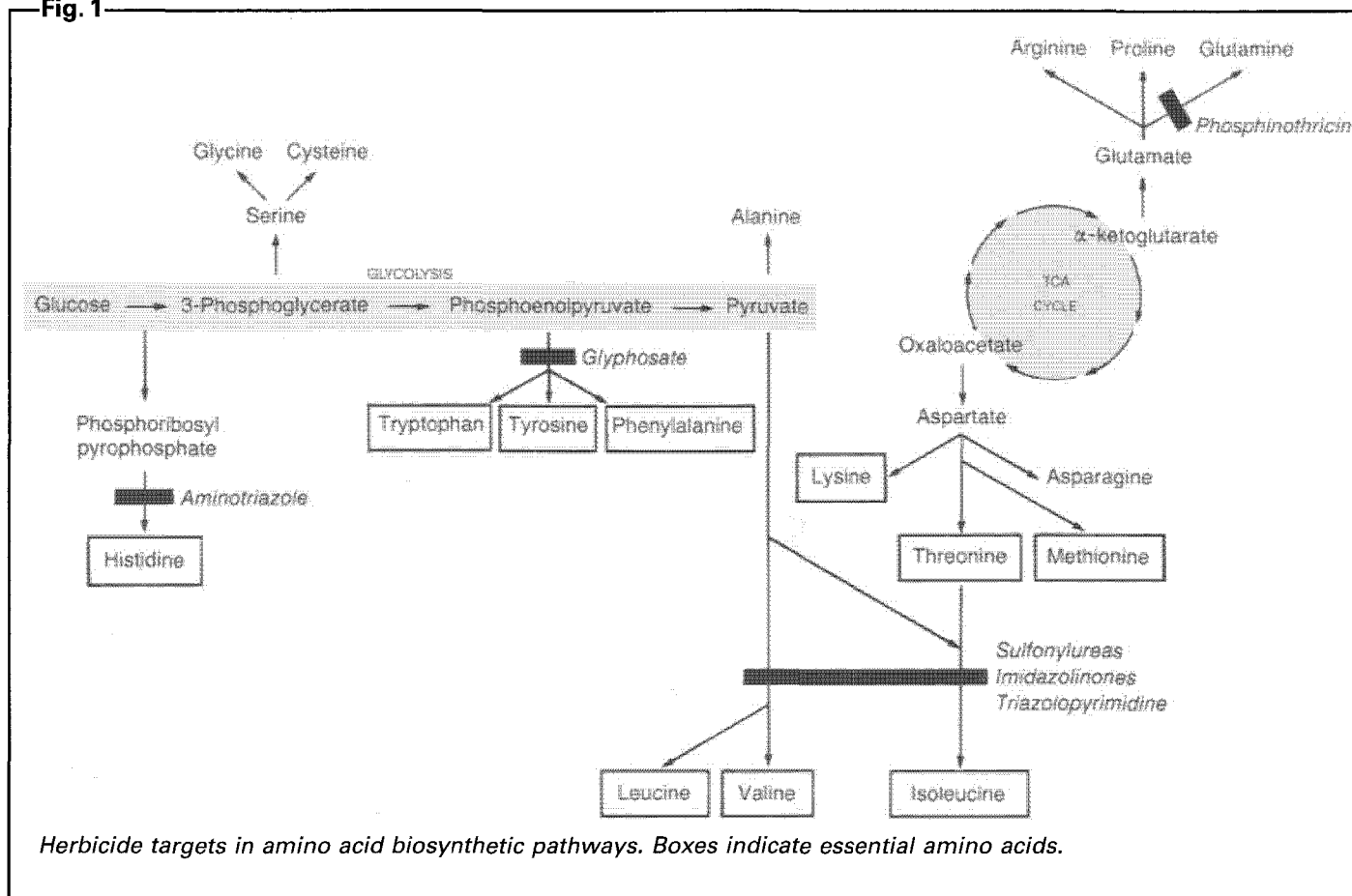
The second strategy adopted was increasing EPSPS activity¹⁶. A cell line of *Petunia hybrida* tolerant to glyphosate was selected in culture; in this line EPSPS is overproduced owing to a 20-fold amplification in the number of copies of the gene present in the nucleus. The over-

producing *Petunia* cells were used to isolate a plant EPSPS cDNA clone, and a chimaeric EPSPS gene was constructed using the CaMV35S promoter and the *Petunia* cDNA. The CaMV35S promoter gives rise to high levels of constitutive expression of coding sequences in all plant tissues. Transformed *Petunia* tissue contained elevated levels of EPSPS and was resistant to four times the glyphosate dose required to kill all the wild-type plants.

- **Phosphinothricin (PPT)** – is an analogue of glutamine. Phosphinothricin acts as a herbicide by inhibiting the amino acid biosynthetic enzyme, glutamine synthase (GS) of plants and bacteria (Fig. 1). In plants, GS is involved in the assimilation of ammonia and in the regulation of nitrogen metabolism. A PPT-tolerant alfalfa cell line had a 10-fold increase in GS activity; this was consistent with the levels of GS-specific RNA and DNA¹⁹. Gene amplification of GS resulted in enhanced GS expression, allowing sufficient enzyme activity even in the presence of the inhibitors. Expression of a mutant alfalfa GS gene in transgenic tobacco plants has also been reported to confer a low level of tolerance to PPT, but only when it is taken up by the roots²⁰. However, converting more glutamate into glutamine might produce undesired effects on the nitrogen metabolism of the plant.

- **Sulfonylureas and imidazolinones** – these broad-spectrum herbicides are notable for their low rates of use and their low toxicity to animals. They inhibit acetolactate synthase (ALS) (Fig. 1). Mutant ALS

Fig. 1



genes, encoding ALS resistant to sulphonylureas and/or imidazolinones, have been found in bacteria, yeast and plants²¹. These mutations have been characterized as single amino acid changes in ALS. Comparison of the amino acid sequences of the plant ALS genes with those of the yeast and three *E. coli* ALS genes showed that they share the same three conserved domains²⁰. Attempts at using microbial genes to generate herbicide-resistant transgenic plants met with two difficulties: first, in plants, ALS is localized in the chloroplast; and second, the bacterial enzyme is composed of two different subunits. Engineering herbicide-resistant plants might thus require expression of the two protein subunits of the enzyme and their translocation into, and assembly within, plant chloroplasts.

Because of these difficulties, the isolation and use of mutant plant ALS genes was thought to be a more direct route for achieving sulphonylurea-resistant transgenic plants. One tobacco gene and one *Arabidopsis* gene have been isolated and sequenced. These genes each carried a single mutation that resulted in a Pro196 to Gln substitution and a

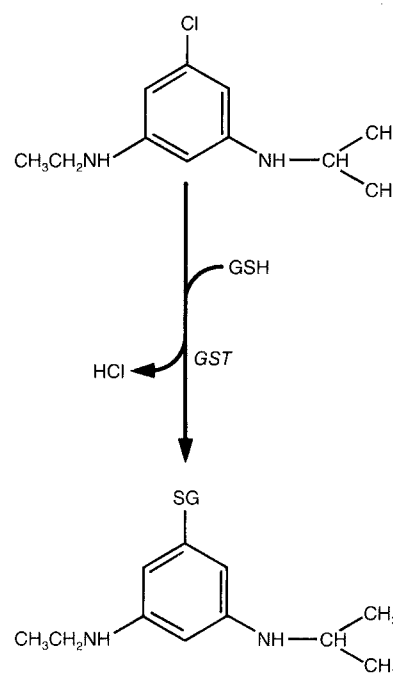
Pro197 to Ser substitution, respectively. Both mutations conferred resistance to sulphonylurea. A second tobacco gene, which contained two mutations resulting in Pro196 to Ala and Trp573 to Leu substitutions, conferred cross-resistance to both sulphonylureas and imidazolinone^{5,22,23}.

Additional mutations in plant ALS genes have been generated by site-directed mutagenesis²⁴. A bacterial expression assay system for the *Arabidopsis* ALS gene was developed to permit the rapid isolation and characterization of the new mutations²⁵. Transgenic herbicide-resistant tobacco plants expressing a mutant ALS gene from tobacco or *Arabidopsis* have been shown to tolerate herbicide concentrations four times that of a typical field application rate^{22,23}.

Introducing a gene encoding an enzyme which will detoxify the herbicide

A second approach to developing herbicide resistance by gene transfer is to identify and clone genes encoding enzymes involved in herbicide detoxification. Manipulations

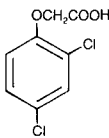
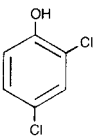
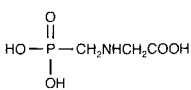
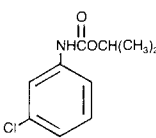
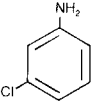
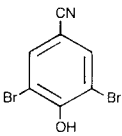
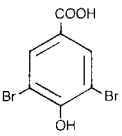
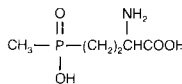
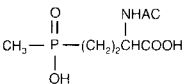
Fig. 2



Detoxification of atrazine by GST. GSH, reduced glutathione; SG, conjugated glutathione; GST, glutathione-S-transferase.

Table 2

Bacterial degradation of herbicides

Herbicide	Primary metabolite	Enzyme(s)	Representative organism(s)
2,4-D 		Monooxygenase	<i>Alcaligenes</i>
Glyphosate 		Phosphonatase	<i>Pseudomonas</i>
Chlorpropham 		Acylamidase	<i>Bacillus</i> <i>Anacystis</i>
Bromoxynil 		Nitrilase	<i>Klebsiella</i>
Phosphinothricin 		Phosphinothricin acetyl transferase	<i>Streptomyces</i>

involving the introduction into plants of additional enzymes that are designed specifically to inactivate the relevant herbicide are considered superior to those aimed at changing the herbicide's target. If the target enzyme is altered, it may not retain full normal function, and secondary phenotypic consequences may be detrimental to the engineered plant. However, the obvious setback with the detoxification approach is the lack of availability of suitable enzymes.

• Herbicide-detoxifying enzymes from plants

Herbicide-detoxifying enzymes such as mixed-function oxidases, amidases, decarboxylases and conjugative systems have been identified in many herbicide-tolerant plants²⁶. The system most extensively studied is the conjugation of xenobiotic compounds with glutathione (Fig. 2). This conjugation is catalysed by multifunctional glutathione-S-transferases (GSTs). Genes encoding atrazine- and alachlor-detoxifying GSTs (GSTI and GSTIII) have been

isolated from maize²⁷, though the introduction and expression of these genes in other plants has met with little success. This is perhaps not surprising; in maize, the active atrazine GST is the product of more than one gene locus, and the glutathione level, in contrast to many other crop species, is very high.

Another class of conjugative enzymes is represented by the *N*-glucosyl transferase present in metribuzin-tolerant tomato²⁸. Detoxification of the herbicide is due to increased activity of *N*-glucosyl transferase. Metribuzin tolerance in tomatoes is controlled by one gene locus. A third class of plant enzymes is the mixed-function oxidases, involved in the detoxification of 2,4-dichlorophenyl acetic acid (2,4-D) in tolerant pea²⁸ and of dicamba in tolerant barley²⁹.

Little information is available on the molecular basis of herbicide detoxification in plants. Consequently, the development of a genetic engineering strategy based on plant herbicide-detoxifying enzymes is slower than certain other approaches.

• Herbicide-detoxifying enzymes from bacteria

Many herbicides are rapidly degraded by soil microflora. Such microorganisms provide an alternative strategy for obtaining herbicide-tolerance genes. Bacterial degradative systems have been extensively studied and a number of detoxifying enzymes have been identified (Table 2).

Engineering herbicide resistance into plants has been achieved by introducing bacterial genes encoding enzymes that inactivate the herbicides by nitril hydrolysis, acetylation or removal of the acetate side-chain.

A strain of the soil bacterium *Klebsiella ozaenae* has been isolated which can use bromoxynil (3,5-dibromo-4-hydroxybenzonitrile, an inhibitor of photosynthetic electron transport) as its sole nitrogen source. It possesses a plasmid-encoded nitrilase gene (*bxn*) that is constitutively expressed and converts the herbicide to its inactive metabolite 3,5-dibromo-4-hydroxybenzoic acid in a single step. The *bxn* gene has been

cloned and introduced into plants³⁰; transgenic tobacco and tomato plants expressing the *bxn* gene were able to tolerate bromoxynil³¹.

The *bar* gene from *Streptomyces hygroscopicus* has been cloned and characterized³². This gene encodes a phosphinothricin acetyl transferase (PAT) which converts PPT into the non-toxic acetylated form. The *bar* gene was introduced into tobacco, potato and tomato; transgenic plants expressing various levels of PAT were fully resistant to the herbicide^{33,34}.

Finally, the gene *tfdA* from the soil bacterium *Alcaligenes eutrophus* has recently been cloned and sequenced³⁵. This gene encodes the enzyme 2,4-dichlorophenoxyacetate monooxygenase (DPAM), which degrades the herbicide 2,4-D to 2,4-dichlorophenol. This metabolite is not toxic to plants, at least up to concentrations of 10 mg l⁻¹, whereas more than 2 mg l⁻¹ of 2,4-D severely reduced growth. The DPAM-coding sequence, fused with plant expression sequences, was introduced into tobacco using *Agrobacterium*-mediated transformation. Transgenic plants were resistant to 2,4-D and were phenotypically normal^{36,37}. Unfortunately, the DPAM gene cannot be used as a selectable marker in leaf disc transformation³⁶; in chimaeric tissue, transformed cells might be overgrown or inhibited by untransformed callus developing rapidly in the presence of 2,4-D.

Conclusion

Engineering herbicide tolerance into crops has proved to be a more complex undertaking than originally envisaged. A large number of compounds can act as herbicides but molecular research has concentrated on those with properties such as low toxicity and high effectivity. Progress in this area during the past five years has been rapid – due, partly, to the development of efficient plant transformation and regeneration systems for certain species. Transgenic plants have now been reported in more than 30 species³⁸. Genetically engineered soybean, cotton, rice, oil-seed rape, corn, sugar beet and alfalfa crops are expected to enter the market by the year 2000. Reliable transformation systems for the major cereal crops are lagging behind, but are hoped to be routinely available in the near future.

Herbicide resistance represents a valuable trait for use both in basic research and in commercial agriculture. There is potential for herbicide-resistance genes to be used as dominant selectable markers, analogous to the antibiotic-resistance genes. In agriculture, weeds reduce productivity by at least 12%, a figure that has increased from 8% since the 1940s. Herbicide-resistant crops could lead to a more cost-effective and environmentally acceptable weed control. In addition, such genetically engineered plants would provide greater flexibility in the choice of crops for rotation or double crop-plantings.

Before final decisions on the commercialization of specific herbicide-tolerant crops can be made, however, factors such as potential loss in vigour and yield, herbicide performance, crop and chemical registration costs, potential for outcrossing to weed species, potential of the crop itself for becoming a weed, and proprietary rights issues must be carefully considered.

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