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# Engineering of Herbicide Resistance in Plants

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## Introduction

In modern agriculture, herbicides are commonly applied to eliminate weeds as they are more labour- and energy-efficient than manual or mechanical weed control. Over a hundred phytotoxic molecules are currently used as commercial herbicides. Most herbicides originate from extensive screening of a large number of synthetic compounds. Those that exhibit both lack of activity towards an important crop, and phytotoxicity towards its major weeds have been chosen for development. In recent years, increasing concern about contamination of the environment, toxicity to animals and persistence of residues in soil and water, have encouraged further research for new herbicides that are highly effective, safe for animals and rapidly biodegraded.

Many herbicides exert their effects by inactivating 'target proteins' (usually enzymes) essential for vital functions such as the photosynthetic or other biosynthetic pathways unique to plants. Because crop plants usually share these processes with competing weeds, many herbicides are non-selective. Others can be used selectively on tolerant crops, mainly as a consequence of a differential uptake or metabolism of the herbicide or by a precise localization of the herbicide application. An alternative is to confer resistance to crops against broad-spectrum herbicides. This would permit the use of such herbicides and selection from this range of compounds of those which are environmentally acceptable and non-toxic.

Basically, two approaches have been worked out for the engineering of herbicide resistance in plants: (1) the modification of the enzyme or other target for herbicidal action in the plants to render it insensitive to the herbicide or by inducing the overproduction of the unmodified target protein, thus permitting normal metabolism to occur in spite of the presence of the herbicide; (2) the introduction of a enzyme or enzyme system to degrade and/or detoxify the herbicide in the plant before it can act. Plants modified in these

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ways may be obtained either by selection for resistance against the herbicide or by applying gene transfer techniques. Genetic engineering of plants may be brought about by the use of gene transfer vectors derived from the soil bacteria *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* or by direct gene transfer of DNA into plant cells or protoplasts.

This review describes the current status of application of the different strategies to introduce resistance in crops against several important herbicides, and future perspectives of crops with herbicide-resistant traits.

## Modification of the target of herbicide action

This approach depends on the identification in molecular terms of the biochemical site of herbicide action in the plant cell. Considerable effort has been devoted to elucidation of the mode of action of herbicides through physiological, biochemical and genetic studies. Some herbicides have been shown to disrupt amino acid biosynthesis pathways (LaRossa and Falco, 1984) (*Figure 1*) and others to interfere with photosynthesis (Arntzen, Pfister and Steinback, 1982). Genes encoding herbicide-sensitive or insensitive target proteins have been isolated from both plants and micro-organisms. The following examples show how they have been used to engineer herbicide tolerance.

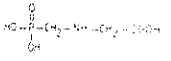
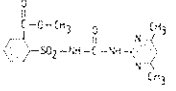
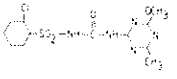
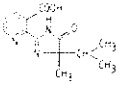
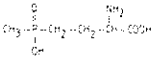
| Herbicide | Active compound | Structure | Inhibited pathway | Target enzyme |
|---|---|---|---|---|
| Roundup | Glyphosate |  | Common aromatic | 5-enolpyruvyl-shikimate-3-phosphate synthase |
| Oust | Sulfometuron methyl |  | Branched-chain | acetolactate synthase |
| Glean | Chlorsulfuron |  | Branched-chain | acetolactate synthase |
| Arsenal | AC 243,997 |  | Branched-chain | acetolactate synthase |
| BASTA | Phosphinothricin |  | Glutamine | glutamine synthetase |

**Figure 1.** Herbicides which inhibit essential amino acid biosynthesis (La Rossa and Falco, 1984): glyphosate; sulfometuron methyl and chlorsulfuron, sulphonylurea herbicides; imazapyr (AC 243, 997), an imidazolinone herbicide and phosphinothricin.

GLYPHOSATE

Glyphosate [*N*-(phosphonomethyl)glycine] is currently the most extensively used non-selective herbicide. Strong evidence has accumulated that glyphosate acts by inhibiting 5-enolpyruvylshikimate-3-phosphate synthase (EPSP) (EC 2.5.1.19), a key enzyme in the biosynthesis of aromatic amino acids in bacteria and plants (*Figure 1*) (*see* Shah *et al.*, 1987).

A gene (*aroA*) coding for a glyphosate-tolerant form of EPSP has been isolated from the bacterium *Salmonella typhimurium* (Comai, Sen and Stalker, 1983). A proline-(101)-to-serine amino acid change in the mutant *aroA* allele yielded an EPSP with decreased affinity for glyphosate (Stalker, Hiatt and Comai, 1985). Mutations at other positions of the EPSP also yielded resistant enzymes (R.T. Fraley, unpublished results). Direct evidence that EPSP is the primary target of the herbicide stems from the observation that increased expression of the *aroA* gene in *Escherichia coli* also conferred resistance to glyphosate (Rogers *et al.*, 1983). The *Salmonella* glyphosate-resistant *aroA* gene was introduced in tobacco (*Nicotiana tabacum*) (Comai *et al.*, 1985) and tomato (*Lycopersicon esculentum*) plants (Fillatti *et al.*, 1987) using chimaeric gene constructs in Ti plasmid vectors and *Agrobacterium*-mediated T-DNA transformation. Expression of the chimaeric *aroA* gene under control of the octopine synthase promoter in regenerated plants resulted in the presence of a bacterial EPSP in the plant cell cytoplasm. Transgenic plants were up to three times more tolerant to glyphosate than the control plants, depending on the level of *aroA* expression.

Glyphosate-tolerant cell cultures of *Corydalis sempervirens* and *Petunia hybrida* were obtained after selection on the herbicide (Amrhein *et al.*, 1983; Steinrucken *et al.*, 1986). The petunia cell line overproduced EPSP as a result of a 20-fold gene amplification. A cDNA clone encoding EPSP was isolated and the nuclear gene was shown to encode a cytoplasmic precursor with an amino-terminal peptide sequence that permits post-translational transfer from the cytoplasm to the chloroplasts (Della-Cioppa *et al.*, 1986). As amino-acid biosynthesis is usually conducted by enzymes encoded by nuclear genes that function in the chloroplasts, it is desired to target the gene product to the chloroplasts. The transfer of foreign proteins encoded in the nucleus to chloroplasts has been demonstrated when the protein is linked to chloroplast-specific transit peptides (van den Broeck *et al.*, 1985). Genomic EPSP synthase genes have been isolated from *Arabidopsis thaliana* and *Petunia hybrida* (Klee, Muskopf and Gasser, 1987; G.S. Gasser and co-workers unpublished results). Two genes are present in *Arabidopsis* and two sets of hybridizing sequences are observed in petunia.

A chimaeric EPSP gene encoding wild-type precursor EPSP under control of the cauliflower mosaic virus (CaMV) 35S promoter was introduced into petunia plants (Shah *et al.*, 1986b). This promoter has been shown to direct high-level expression of foreign genes in transformed plants (Odell, Nagy and Chua, 1985). Transgenic plants were significantly tolerant to glyphosate and showed increased production of EPSP that was localized in the chloroplasts. Similarly, a gene fusion between the transit peptide sequence of the petunia

cDNA clone and an *E. coli* gene which encodes a highly glyphosate-resistant EPSP yielded transgenic tobacco plants that showed higher tolerance to glyphosate than plants over-expressing the wild-type EPSP gene (Della-Cioppa *et al.*, 1987; Fraley *et al.*, 1987).

### SULPHONYLUREAS AND IMIDAZOLINES

The sulphonylurea herbicides inhibit the acetolactate synthase (ALS) (EC 4.1.3.18) from bacteria, yeast or plants (Chaleff and Mauvais, 1984; Ray, 1984; Falco and Dumas, 1985). ALS is involved in the biosynthesis of the branched-chain amino acids leucine, isoleucine and valine. An unrelated class of herbicides, the imidazolinones, also inhibit ALS (Shaner, Anderson and Stidham, 1984; Muhitch, Shaner and Stidham, 1987) (*Figure 1*). Dominant mutants that confer resistance to sulfometuron methyl in *Salmonella typhimurium* (LaRossa and Schloss, 1984), *Saccharomyces cerevisiae* (Falco and Dumas, 1985), *Escherichia coli* (Yadav *et al.*, 1986) and *Chlamydomonas rheinhardii* (Hartnett, Newcomb and Hodson, 1987) have been isolated. These mutations map to the structural genes for ALS. Nucleotide sequences of mutant genes revealed single nucleotide differences. The encoded enzymes carry single amino acid substitutions (*Table 1*) and vary in ALS activity and in herbicide sensitivity (Yadav *et al.*, 1986).

Chlorsulfuron- or sulfometuron-resistant plants have been obtained by selection in cultures of haploid tobacco (*Nicotiana tabacum*) protoplasts (Chaleff and Ray, 1984) and in mutagenized *Arabidopsis thaliana* seedlings (Haughn and Somerville, 1986). In contrast to the situation with glyphosate, overproduction of the target enzyme has not been reported in plants resistant to sulphonylurea herbicides. Diploid tobacco plants were regenerated and the resistant phenotype was shown to be expressed at the whole-plant level. Through genetic crosses it was established that single nuclear mutations in two unlinked loci were cosegregating with a herbicide-insensitive ALS activity (Chaleff and Mauvais, 1984; Chaleff and Bascomb, 1987). A single dominant nuclear mutation at one locus in *Arabidopsis* conferred resistance (Haughn and Somerville, 1986).

However, not all resistant phenotypes against sulphonylureas were correlated with a modified ALS. Field screening has yielded chlorsulfuron-resistant alfalfa seedlings (*Medicago satvia*) (Stannard and Fay, 1987) and mutagenized soybean seeds (*Glycine max*) have been selected for chlorsulfuron tolerance (Sebastian and Chaleff, 1987). In both cases, none of the mutants were resistant at the level of the ALS enzyme activity. In the soybean mutants, tolerance was conditioned by a single recessive gene. In other studies, selection on haploid anther cells and on protoplasts derived from these microspores of *Brassica napus* yielded chlorsulfuron-resistant mutants. Only some of the regenerated ALS mutants were resistant at the level of ALS (Swanson *et al.*, 1988).

The genes encoding the wild-type and mutant ALS have been isolated from *N. tabacum* and from *A. thaliana* (Mazur, Chui and Smith, 1987; Haughn *et al.*, 1988; Lee *et al.*, 1988). The tobacco HRA line, which is mutated at the *SURB*

**Table 1.** Mutant target sites

| Herbicide | Gene locus | Organism | Mutation | Reference |
|---|---|---|---|---|
| A. Glyphosate | *aroA* | *Salmonella typhimurium* | Pro101 → Ser | Comai, Sen and Stalker, 1983 |
| B. Sulphonylureas | *ilvGM* | *Escherichia coli* | Ala26 → Val | Yadav *et al.*, 1986 |
| | *ILV2* | *Saccharomyces cerevisiae* | Pro192 → Ser | Yadav *et al.*, 1986 |
| | *SURB-Hra* | *Nicotiana tabacum* | Pro196 → Ala | Lee *et al.*, 1988 |
| | | | Trp573 → Leu | |
| | *SURA-c3* | *Nicotiana tabacum* | Pro196 → Gln | Lee *et al*, 1988 |
| | *csr1* | *Arabidopsis thaliana* | Pro197 → Ser | Haughn *et al.*, 1988 |
| C. Phosphinothricin | GS | *Medicago sativa* | Gly245 → Ser, Cys, Arg | H.M. Goodman, unpublished results |
| | | | Arg332 → Lys | |
| D. Triazines | *psbA* | *Amaranthus hybridus* | Ser264 → Gly | Hirschberg and McIntosh, 1983 |
| | | *Solanum nigrum* | Ser264 → Gly | Goloubinoff and Edelman, 1985 |
| | | *Chlamydomonas rheinhardii* | Ser264 → Ala | Erickson, Rahire and Rochaix, 1985 |
| | | | Phe255 → Tyr | Barber, 1987 |
| | | | Val219 → Ile | |

locus and a second tobacco line, C3, which carries a mutation at the *SURA* locus, have been used as source of mutant ALS genes. Four genes, representing all the ALS loci from the two mutant lines have been isolated (Falco *et al.*, 1987; Lee *et al.*, 1988). A single ALS gene is present in *A. thaliana* (Mazur, Chui and Smith, 1987). A mutant ALS gene, *crs1*, has been isolated from *Arabidopsis* (Haughn *et al.*, 1988). The plant ALS genes are nuclear encoded and contain a chloroplast transit peptide at the amino-terminal end. In resistant tobacco and *Arabidopsis* ALS genes, different sites in the protein chain where mutations occurred have been observed as summarized in *Table 1*. For example, the proline-to-serine substitution by a single nucleotide change in the *Arabidopsis* ALS gene (position 196) and the proline-to-alanine substitution in *SURB-Hra* tobacco gene occur at the same position as a mutation which confers resistance in the yeast ALS gene (position 192) (Yadav *et al.*, 1986; Haughn *et al.*, 1988). The double mutation in the *SURB-Hra* gene yields an enzyme which is more resistant to sulphonylureas (Lee *et al.*, 1988). Reintroduction of mutant genes isolated from either locus of tobacco conferred resistance in transformed cells of tobacco and tomato. Plants regenerated from cells transformed with the *SURB-Hra* gene are resistant to field application rates of chlorsulfuron (Falco *et al.*, 1987; Lee *et al.*, 1988). Similarly, tobacco plants transformed with the *Arabidopsis crs1* gene were resistant to chlorsulfuron (Haughn *et al.*, 1988).

Tolerance to imidazolinones has been demonstrated in maize (*Zea mays*) cell lines that were selected in tissue cell culture. The resistant phenotype was expressed in regenerated plants from these resistant lines and was also attributable to an altered ALS enzyme (Shaner and Anderson, 1985). Plants homozygous for the resistant gene were tolerant to 300 times higher concentrations of several imidazolinone herbicides (Anderson and Georgeson, 1986). This is the first example of selection and regeneration of herbicide-tolerant lines in monocotyledonous crops such as maize. A major drawback has been the inability to regenerate plants from monocot tissue culture (Rhodes, Lowe and Ruby, 1988; Rhodes *et al.*, 1988).

Cross resistance of mutants to sulphonylureas and imidazolinones might be related to the origin of the herbicide-binding site of ALS (Schloss, Ciskanik and Van Dyk, 1988). It has been observed in the imidazolinone-resistant maize (Shaner and Anderson, 1985) but selective resistance of the products might be possible (Haughn and Somerville, 1986).

## L-PHOSPHINOTHRICIN (PPT)

L-Phosphinothricin (PPT) is an analogue of glutamate which was initially characterized as part of a tripeptide antibiotic produced by *Streptomyces viridochromogenes* (Bayer *et al.*, 1972). It is an irreversible inhibitor of glutamine synthetase (GS) (EC 6.3.1.2) in bacteria and in plants (Colanduoni and Villafranca, 1986; Manderscheid and Wild, 1986). Two forms of GS with a cytosolic or chloroplastic compartmentalization have been identified in plants. GS is involved in the primary assimilation of ammonia produced by nitrate reduction or nitrogen fixation in the roots as well as the reassimilation of ammonia released by photorespiration in plants. In higher plants PPT causes

rapid accumulation of ammonia and inhibits photosynthesis which leads to death of the plant cell (Tachibana *et al.*, 1986). A PPT-tolerant alfalfa (*Medicago sativa*) suspension culture line was selected on increasing PPT concentrations (Donn *et al.*, 1984). Gene amplification of GS resulted in a fivefold enhanced GS expression, yielding a cell line which is about twenty times more resistant than the parental line. Further screening showed that the copy number of the amplified gene was correlated to the level of resistance. A GS cDNA clone was isolated from the tolerant line (Tischer, Das Sarma and Goodman, 1986) and expression of this GS gene under control of the CaMV 35S promoter confers low-level tolerance to PPT in transgenic tobacco plants (Eckes and Wengemayer, 1987). It has also been shown that this plant GS gene complements a mutant GS gene (*glnA*) in *Escherichia coli* (Das Sarma, Tischer and Goodman, 1986), which can be used to select for resistant plant GS mutants in *E. coli*. Specific mutations which result in PPT resistance are a glycine to serine, cysteine or arginine at position 245, or arginine to a lysine at position 332 (H.M. Goodman, unpublished results).

## TRIAZINES

The *S*-triazine-type herbicides such as atrazine and the chemically unrelated ureas such as diuron inhibit photosynthesis (Arntzen, Pfister and Steinback, 1982). The mechanism of action involves the binding of the herbicide to chloroplast thylakoid membranes and blocking of electron transport at the second electron acceptor of photosystem II. This protein, the $Q_B$ protein, is encoded by the *psbA* gene in the chloroplast genome (Zurwarski *et al.*, 1982). It is expressed as a precursor form which is processed to a 32 kd mature form in the thylakoid membranes (Mattoo and Edelman, 1987). Some crops such as maize are resistant to atrazines because they can degrade the herbicide (*see* page 330). In agricultural areas where atrazine has been extensively used, atrazine-resistant biotypes of many weed species have appeared. Resistance was found to be maternally inherited and was correlated with mutations in the *psbA* gene (Souza Machado and Ditto, 1982).

The *psbA* sequence of a resistant weed, *Amaranthus hybridus*, revealed three nucleotide differences. Two mutations were silent, whereas the third mutation resulted in a serine-to-glycine change at residue 264 (Hirschberg and McIntosh, 1983). Mutant *psbA* genes isolated from other atrazine-resistant weeds showed a highly conserved sequence among different species and revealed single amino acid substitutions at the same position in the protein chain (Barber, 1987; Goloubinoff and Edelman, 1984). These mutations resulted in an at least tenfold slower flow of electrons through this protein. From the more than 50 weed species that evolved resistance to atrazine, only *Abutilon* has been shown to possess a degradation pathway as a mode of resistance (Andersen and Gronwald, 1987). A mutation conferring resistance in the blue-green alga *Chlamydomonas rheinhardii* was caused by a serine-to-alanine change at position 264 of the *psbA* coding sequence (Erickson, Rahire and Rochaix, 1985). A diuron-resistant mutant has a isoleucine-to-valine change at position 219. These algae mutants exhibited

varying cross resistances towards different photosystem-II-inhibiting herbicides (Galloway and Mets, 1984). Similarly, naturally occurring weed biotypes with varying atrazine resistance levels showed variable cross resistances (Arntzen, Pfister and Steinback, 1982).

Although genes encoding atrazine-resistant $Q_B$ proteins are available, the engineering of atrazine-resistant plants has not been possible, primarily because of the lack of reproducible systems for transformation of plant chloroplasts (Cornelissen *et al.*, 1987). Nuclear transformation of a mutant *psbA* gene carrying a transit peptide-encoding sequence of a nuclear gene yielded atrazine-tolerant transgenic tobacco plants (Cheung *et al.*, 1988).

The maternally inherited triazine resistance from two weeds has been used for introducing the trait into related crop varieties. The triazine-tolerant trait was transferred from a resistant weed biotype of *Brassica campestris* to cultivated rapeseed cultivars from *Brassica napus* and *Brassica campestris* (Beversdorff *et al.*, 1980). Resistance from *Setaria viridis* was crossed into the crop *Setaria italica* (Darmency and Pernes, 1985). However, many of the atrazine-tolerant commercial lines fail to perform agronomically at levels comparable to non-tolerant lines (Souza Machado, 1982). Although it is not clear if a slower flow of electrons decreases photosynthesis, there is generally a 20% loss of yield and a considerably lower competitive fitness (Gressel and Ben-Sinai, 1985; Vaughn, 1986; Ricroch *et al.*, 1987).

## CONCLUSION

Although these examples illustrate how modified targets or an increased expression of a target might confer tolerance to a herbicide, some factors have to be taken into account. To reduce the complexity of generating such engineered plants, the action of the herbicide is preferentially confined to a unique target. As an altered affinity of the target for the herbicide is required without a concomitant decrease in activity, the generation of a mutant form is probably a rare event. This is presumably achieved only by a very specific genetic alteration. In some cases mutant target enzymes are found (e.g. ALS) or amplified genes are generated (e.g. GS and EPSP synthase) (Amrhein *et al.*, 1983; Donn *et al.*, 1984; Smith, Pratt and Thompson, 1986; Steinrucken *et al.*, 1986). The latter two cases represent the first gene amplification events described in plants. Single-site mutations are clearly the easiest to obtain, both in plants and micro-organisms. The frequency of resistance mutations versus gene amplification events might be totally dependent on the characteristics of the enzyme system. For example, in multimeric enzyme complexes such as GS which contains eight subunits, a selection probably favours the isolation of mutants in expression level, rather than structural mutants. Resistant mutants can be isolated after selection on whole plants or in cell culture. Selection on whole plants is difficult because the population size becomes a limiting factor. Cell culture systems permit the screening of a large number of cells. However, only a limited number of crop species can be regenerated from cell cultures. An interesting selection system is available for the fern *Ceratopteris*. Selection pressure is applied on the photoautotrophic haploid gametophytes (Hickok,

1987). Expression of the selected trait can be monitored in both haploid gametophytes and in diploid vascular sporophytes, which are produced by fertilization of selected gametophytes. For example, monogenic paraquat resistance has been described. Such mutants can be used for isolation of the resistance genes. Resistance is often achieved in 'non-cultivar' material and must be transferred to commercial lines. This requires several back-crosses to introduce the resistance in suitable breeding material. Even with two generations per year, it takes at least 5 years before a herbicide-resistant variety can be field tested. It is clear that biotechnologists will have to team up with breeders (Reid, 1987).

A great advantage of a genetic engineering approach is that the gene can be used to transform different plant species. In order to apply plant transformation, the gene encoding the target has to be available, and transformation procedures for the crop in question are required. Since target components are frequently compartmentalized within the cell or since dual pathways in separate plant cell components may occur, a correct expression of the herbicide-resistance determinant is difficult. Moreover, the wild-type gene cannot easily be deleted from the plant genome, and consequently, a mixture of wild-type and mutant gene products in transgenic plants may yield an inconsistent resistance phenotype.

## Detoxification or degradation of the herbicide

The second approach for developing herbicide resistance by gene transfer involves enzymes that inactivate the herbicide before inhibition of the plant-cell target site. Herbicide-detoxification or herbicide-degradation pathways may occur in plant species tolerant to specific herbicides or have been observed in micro-organisms. In two cases resistance genes isolated from micro-organisms have been used to produce herbicide-resistant crops.

### PLANT DETOXIFYING ENZYMES

The observation that many crop species are tolerant to specific herbicides encouraged the use of these herbicides for the selective control of weeds by exploiting the difference in phytotoxicity without significantly reducing crop yields. Herbicide selectivity in many crops is determined by several factors such as rates of absorption in the plant, translocation, subcellular localization, variation in target site sensitivity and metabolic detoxification to non-phytotoxic derivatives (Jensen, 1982). The selective properties of herbicides often result from a complex interaction of these factors. Differential metabolism probably constitutes the most important factor and genes encoding the enzymes responsible might be explored as herbicide-resistant determinants (Cole, Edwards and Owen, 1987; Owen, 1987). A variety of enzymes such as mixed-function oxidases, amidases, decarboxylases, and thiol-, sugar- and amino acid-conjugative enzyme systems were identified in herbicide-tolerant plant species. Some mechanisms involve the hydroxylation of aryl groups, oxidation of alkyl substituents or sulphur moieties, hydrolysis of carboxylic

acid ester groups, reductive deamination and glycosidation (Owen 1987). Mechanisms by which plant species transform foreign compounds are reviewed by Cole, Edwards and Owen (1987).

A well-known example is the detoxification of atrazine and alachlor in tolerant maize lines which involves conjugation with the tripeptide glutathione by the enzyme glutathione-*S*-transferase (EC 2.5.1.18) (Shimabukuro *et al.*, 1971). The conjugate is non-toxic to the plant cell and is eventually further metabolized. The enzyme is present at high levels and is encoded by a multigene family (Moore *et al.*, 1986; Shah *et al.*, 1986a; Grove *et al.*, 1988). In metribuzin-tolerant tomatoes, detoxification involves conjugation with UDP glucose by an *N*-glucosyl-transferase (Frear *et al.*, 1983). Tolerant tomato cultivars contain more transferase activity than sensitive lines, and tolerance is controlled by one gene locus (Souza Machado and Ditto, 1982). Mixed-function oxidases are involved in the detoxification of 2,4-dichlorophenoxyacetic acid (2,4-D) in pea (*Pisum sativum*) and of dicamba in tolerant barley (*Hordeum vulgare*) (Comai and Stalker, 1986). Some herbicides, such as the bipyridinium herbicide paraquat, are known to generate active oxygen species which lipoxidize plant membranes, causing death of the plant cell. Enhanced levels of the enzymes that detoxify oxygen radicals might be correlated with resistance to such herbicides. At least three enzymes—a superoxide dismutase, a catalase and a peroxidase—are probably involved in resistance against paraquat in *Conyza* species (Shaaltiel and Gressel, 1986, 1987).

In some cases corresponding genes have been isolated but, as most examples deal with multienzyme systems, engineering crops is not straightforward.

## BACTERIAL DETOXIFYING ENZYMES

Several soil micro-organisms that are involved in herbicide degradation have been characterized as potential sources of herbicide-resistance genes. Micro-organisms that degrade herbicides are often found in herbicide-contaminated soils. For example, *Pseudomonas, Arthrobacter, Alcaligenes* and *Acinetobacter* species that metabolize 2,4-dichlorophenoxyacetic acid (2,4-D) have been isolated (Don and Pemberton, 1981). The pathway for 2,4-D metabolism and related cholorophenolic compounds has been found to be plasmid-encoded and some genes have been cloned (Amy *et al.*, 1985; Ghosal *et al.*, 1985). Conversion of 2,4-D requires the gene products of three bacterial genes. A *Pseudomonas* species, a *Flavobacterium* and an *Arthrobacter* sp. have been isolated which utilize glyphosate as sole phosphate source (Balthazar and Hellas, 1986; Kishore and Jacob, 1987; Pipke *et al.*, 1987). The metabolic pathway of glyphosate has been characterized (Rueppel *et al.*, 1977) but genes encoding glyphosate-degrading enzymes have not yet been cloned.

A successful approach has been followed for engineering resistance towards the benzonitrile herbicides bromoxynil and ioxynil, which are inhibitors of photosynthetic electron transport. From soil samples enriched in bromoxynil, a *Klebsiella pneumoniae* subsp. *ozaenae* was isolated which could rapidly metabolize bromoxynil and use it as a sole source of nitrogen (McBride, Kenny

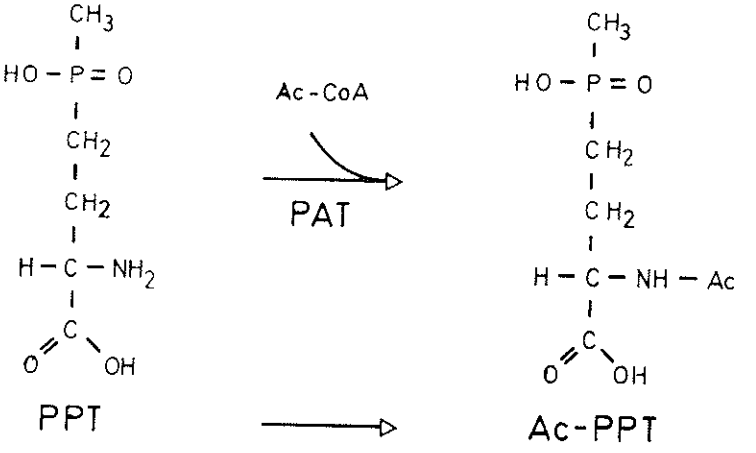



**Figure 2.** Enzymatic reaction of phosphinothricin (PPT) acetyl transferase. An acetyl group from acetyl CoA is transferred on to the amino group of PPT.

and Stalker, 1986). A bromoxynil-specific nitrilase that acts on the cyano group of the molecule converts the herbicide to the metabolite 3,5-dibromo-4-hydroxybenzoic acid. From metabolism studies in tolerant wheat species it is known that this metabolite is at least 100-fold less toxic to plant cells than bromoxynil. The nitrilase was plasmid encoded and the gene *bxn* was constitutively expressed in *K. ozaenae* and *E. coli* and shows a high specificity for bromoxynil as substrate (Stalker and McBride, 1987). Transgenic tobacco and tomato plants that expressed the nitrilase enzyme were resistant to bromoxynil (Stalker, McBride and Malyi, 1988).

Detoxification enzymes can also be found in biosynthetic pathways of naturally produced components with herbicide activity. L-Phosphinothricyl-L-alanyl-L-alanine (bialaphos) is a tripeptide antibiotic produced by *Streptomyces viridochromogenes* (Bayer *et al.*, 1972) and *Streptomyces hygroscopicus* (Kondo *et al.*, 1973). It consists of L-phosphinothricin (PPT) and two L-alanine residues. Upon removal of these residues by peptidases the herbicidal component PPT is released. In the bialaphos biosynthetic pathway, a resistance gene (*bar*) was isolated and characterized (Murakami *et al.*, 1986). It encodes an acetyl transferase (PAT) that acetylates the free amino group of PPT (*Figure 2*) or intermediates in the pathway and protects the micro-organism from autotoxicity (Thompson *et al.*, 1987). Purified PAT enzyme showed high substrate specificity for PPT. The *bar* gene under control of the CaMV 35S promoter was expressed in regenerated transgenic tobacco, potato (*Solanum tuberosum*) and tomato plants (De Block *et al.*, 1987). Greenhouse-grown plants showed complete resistance towards doses of PPT and bialaphos up to ten times the doses recommended by the manufacturer. The resistance was reproducible in field-tested tobacco (*Figure 3*) and potato plants. Two tobacco lines with a 100-fold difference in *bar* gene expression were both resistant to high herbicide levels and there was no significant effect on yield, as measured

by leaf length (Leemans *et al.*, 1987; W. De Greef and co-workers, unpublished results). The fate of the acetylated metabolite in transgenic plants requires careful examination.


**Figure 3.** Evaluation of herbicide resistance in transgenic tobacco plants under field conditions. (a) Untransformed tobacco plants sprayed with 20 l/ha Basta; (b) Transgenic tobacco plants sprayed with 20 l/ha Basta. The pictures are taken 30 days after treatment and show one plot of a factorial design.

CONCLUSION

The use of herbicide-resistance determinants involved in detoxification offers some advantages over target modification. This strategy is preferred when the biochemical site of action of a herbicide is not known or when the target is difficult to engineer because of compartmentalization within the cell, or because of the existence of dual pathways. A few criteria are required: the detoxifying enzyme should be encoded by a single gene or a few genes; the enzyme should not require complex co-factors for its activity; the residual metabolite(s) should have no phytotoxicity and they should eventually be further metabolized. One possible source is the detoxification systems observed in plants. In general, little is known about the molecular basis of herbicide-detoxification mechanisms in plants. This differential metabolism is exploited for herbicide selectivity in some crops, but is restricted to the crops in question. Generally, the herbicide metabolism is only partially elucidated and it is not known whether conversion of the herbicide to its primary metabolite is sufficient or whether the compound must be detoxified further to confer herbicide tolerance. If a plant has a low level of a herbicide-degrading enzyme system, it is conceivable that this level can be increased by selection techniques. Transfer of a plant detoxifying pathway by breeding or genetic engineering has not yet been demonstrated.

An alternative to the use of plant detoxifying or degrading enzymes is use of those from micro-organisms. Indeed, bacteria are easily amenable to genetics and recombinant DNA technology. Elucidation of metabolic pathways is much simpler, and bacterial genes can be properly expressed in plants. Two successful examples have been worked out to engineer herbicide-resistant plants: they took advantage of easily accessible bacterial detoxification enzymes, the performance of which should be independent from the crop species. However, the fate and toxicology of the metabolized herbicide will require careful examination.

## Perspectives

In the last few years, rapid progress in conferring herbicide resistance on crops has been documented, so that the number of scientific papers has at last started to rival the number of reviews on this subject. Field trials over the next two or three years with engineered crops will teach us more of the practical value of the scientific results which have been summarized here.

At present, gene transfer systems for higher plants based on *Agrobacterium* T-DNA transformation or on free DNA transfer enable transgenic plants to be obtained for about 20 crops (Fraley, Rogers and Horsch, 1986). It can be expected that in the near future all major crops will be accessible to improvement using this technology. As the methods for gene transfer and for incorporation of agronomically useful traits in commercial germplasm become routine, the engineering of selective herbicide tolerance will become an accepted and essential strategy and influence weed control practices. Moreover, ever-increasing knowledge of herbicide mode of action and

selectivity and the identification of herbicide-detoxifying enzymes will stimulate genetic manipulation of crops and increase the use of such herbicides. It will also affect the classic procedure followed to develop new herbicides. An integrated research programme will involve the engineering of herbicides, the engineering of targets and the characterization of detoxifying enzymes.

The herbicide-resistance genes can also be used as dominant selectable markers for both basic research and plant breeding. For basic research, the resistance genes will serve as analogues of the antibiotic resistance genes. For plant breeding, the resistance genes can be physically linked to other genes conferring agronomically useful traits which are not easy to detect during transformation and regeneration procedures. They are introduced in plants via transformation using selection for resistance to the herbicide. Subsequently, in a plant breeding programme the trait can be transferred to various cultivars through standard genetic crosses by following the easily assayable herbicide resistance phenotype associated with the linked genetic marker.

Finally, before herbicide-resistant plants can be commercialized, various factors have to be taken into consideration:

1. The performance, the cost and environmental acceptability of the herbicide. When herbicides with well-elucidated weed-control properties and toxicology are dealt with and if it can be shown that residues of the herbicide match those found in crops where the herbicide is already used, registration requirements can be simple.
2. The nature of the resistance mechanism and the potential undesirable agronomic characteristics associated with the resistance trait itself. Most herbicide-resistance traits being developed are for herbicides with single enzyme sites of action. This might lead to an increase of the use of herbicides with single target sites. The development of weed resistance to a specific herbicide by mutation or natural selection can be expected to be highest for this class of herbicides. An additional problem is that crops potentially have the ability to cross genetically with closely related weed species. The risk of inter- and intraspecific transfer of herbicide tolerance should thus be evaluated.
3. The accessibility to commercial germplasm and the time period required to introduce a resistance gene into a significant percentage of the germplasm for a given crop.
4. The quality and quantity characteristics of the engineered crop compared with other competitive products. It will be justified if the manipulated crop solves an uncontrolled weed problem or brings additional benefits to the crop.
5. Given the time periods required from feasibility demonstration in the laboratory to the marketable crop, an important factor will be patent life and the post-patent price for a particular herbicide; indeed, the best cost-effective weed control is obtained with herbicides that are off-patent.
6. The registration procedure and cost of a herbicide on an engineered resistant crop will depend largely on the final destination of the crop: food or non-food. Most naturally resistant crops degrade the selective herbicides and no residues appear in the consumed portions. Regulatory

issues claim that there should be no pesticide residues in foods, even if they are non-toxic. In engineered plants, the herbicide may remain unchanged. For example, there is no information available on the fate of glyphosate in resistant plants (Gressel, 1988).

7. Crops can pose serious problems as volunteer weeds especially in crop rotation programmes. For example, maize appearing in a soybean field has to be considered as a weed. Herbicide-tolerant cultivars might show increased problems as volunteer weeds.

Generally concluding, herbicide-resistant cultivars will not cause a major green revolution, but niches will be created where particularly troublesome weeds cannot be controlled by existing herbicides, or in minor crops with high added value for which no adequate herbicides are developed. It would also be imprudent to invest in herbicide-tolerant cultivars for compounds, the future of which is in doubt on the basis of their toxicology, e.g. triazines and bipyridinium herbicides. On the other hand, the withdrawal of a range of herbicides could leave important gaps in classic chemical weed control which might be covered by herbicide-resistant cultivars. This technology will accelerate the trend of herbicide market evolution to a mature industry, commercializing fewer but more effective, less expensive and environmentally more acceptable products.

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