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THE DEVELOPMENT OF HERBICIDE RESISTANT CROPS

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INTRODUCTION

Genetics in Agriculture

The impressive increases in crop productivity achieved over the last several decades have resulted from genetic improvements in crop cultivars and from advances in agricultural technology and management practices. The nongenetic improvements have included improved weed, disease, and insect control through the use of crop protection chemicals, as well as better mechanization, increased supplies of water and nitrogen, and optimization of planting densities.

The introduction of genetic improvements into crops through breeding, a part of agriculture for thousands of years, was more an art than a science until

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the work of Mendel revealed the rules of inheritance. Genetic engineering in agriculture, which in a broad sense refers to any practice that leads to the development of improved cultivars, began with the application of this knowledge. Genetic traits have been introduced into modern cultivars that improve their insect and disease resistance, harvesting and processing qualities, adaptation to particular environmental conditions, yield (through traits such as heterosis and lodging resistance), and nutritional qualities (through the development of low-glucosinilate and low–erucic acid lines of oilseed rape).

To accelerate and improve crop breeding processes further, a number of new genetic approaches that require increasing degrees of technological sophistication have been developed in the last decade. These methods have included improved seed and pollen mutagenesis techniques, mutagenesis of plant cells in culture, regeneration of plants from cultured cells, plant protoplast fusion, and plant transformation. Mutagenesis procedures have been used to generate new desirable traits, while protoplast fusion has provided a mechanism for moving preexisting desirable traits into crops across species barriers. These approaches have required that efficient screens or genetic selections be developed in order to identify the rare cells expressing the new trait.

With the advent of recombinant DNA technology, it has become possible to transfer specific and well-characterized traits across the broadest evolutionary boundaries. As a result, the term genetic engineering has been reserved for the isolation, amplification, and in vitro manipulation of genes. Transformation methodologies have enabled the subsequent reintroduction of the genes into living cells or organisms. Although a number of technological hurdles remain, plant transformation will clearly be a powerful and broadly applicable methodology that will make crop breeding faster, more predictable, and more far-reaching.

For a number of technical and practical reasons, resistance to herbicides was among the first traits to which these new genetic approaches were applied. In some cases, a specific target of herbicide action had been identified through physiological and biochemical studies. In other cases, genetic studies had shown that resistance to a herbicide was a dominant trait exhibiting the simple Mendelian inheritance pattern of a mutation in a single nuclear gene. Dominance makes genetic selection of herbicide-resistant mutants or transformants easier. The potential utility of herbicide-resistance genes as dominant selectable genetic markers for research in plants, in a manner analogous to that for antibiotic-resistance genes in bacteria, has provided an incentive for research. In addition to these technical considerations, the agronomic importance of herbicides has been a major driving force behind the development of herbicide-resistant plants.

Here we review recent work in the development of herbicide-resistant crops. A list of the herbicides for which these efforts have been initiated, along with their structures and their primary targets, is given in Table 1. We present examples of the introduction of herbicide resistance into crop plants by several different techniques, the status of development of herbicide-resistant cultivars, and future prospects for such work. A number of recent reviews have covered mechanisms of herbicide action (21, 61, 64) and the genetic engineering of herbicide-resistant plants (13, 89, 104).

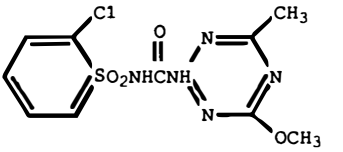
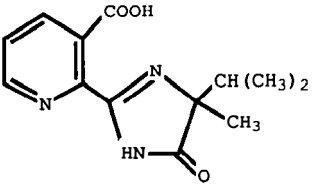
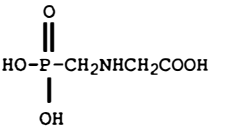
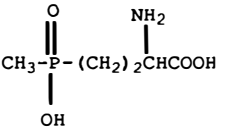
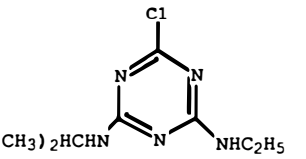
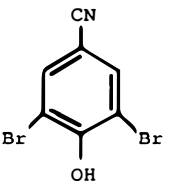
Herbicides in Agriculture

Even those whose experience with weeds has been limited to hand cultivating a home garden can appreciate the usefulness and efficiency herbicides provide for commercial agriculture. Herbicide treatments are an integral part of modern agriculture because they provide cost-effective increases in agricultural productivity. Increased yields result from reduced weed competition for water, light, and nutrients. In addition, crop quality often improves in the absence of contaminating weed seeds, such as wild mustard seeds in harvested canola or wild garlic seeds in wheat. (Weed seed contamination can result in off-flavors following processing, and therefore in reduced remuneration for the grower.) Herbicides can also aid soil conservation efforts through no-till agricultural practices, wherein herbicides rather than tillage are used to reduce weed populations prior to planting.

Herbicides have traditionally been discovered by screening novel compounds in a series of increasingly specific tests. Compounds are first tested for activity against a spectrum of weeds and lack of activity against targeted crops. Promising compounds are further tested in more extensive greenhouse screens and finally in small-scale field trials. Development candidates must then run the gauntlet of acute and chronic toxicology tests. To be commercially successful, herbicides must have potent biological activity against a broad spectrum of weeds and at the same time be nontoxic to crop plants, mammals, and invertebrates; have relatively short soil residual properties; and have favorable production costs. It is becoming increasingly difficult to identify new compounds that meet these criteria and can compete with the many excellent existing products. In the 1950s about 1 in 2000 screened compounds were commercialized; by the 1970s, the rate had dropped to approximately 1 in 7000 compounds, while in the 1980s hardly 1 in 20,000 compounds emerged from these screens.

Selective toxicity of herbicides to weeds but not to crops is one of the most difficult properties to achieve, as might be expected from the biological relatedness of weeds and crops. Selectivity is a function of the physico-

Table 1 Herbicide structures & targets

Herbicide	Inhibited Pathway	Primary Target	Structure
Chlorsulfuron (Sulfonyl-urea)	Branched-chain amino acid biosynthesis	acetolactate synthase	
Imazapyr (Imidazo-linone)	Branched-chain amino acid biosynthesis	acetolactate synthase	
Glyphosate	Aromatic amino acid biosynthesis	5-enolpyruvyl-shikimate-3-phosphate synthase	
Phosphino thricin	Glutamine biosynthesis	glutamine synthase	
Atrazine (Triazine)	Photosynthesis	Q_β protein	
Bromoxynil	Photosynthesis	Q_β protein	

chemical properties of a compound, and of the biochemical interactions of the compound with the crop and the weeds. For example, herbicides that do not percolate beyond the top soil layer, and thus do not affect crop roots that extend below this layer, can provide selectivity. Environmental conditions such as climate, soil pH, and soil organic content influence these interactions. For some compounds, management practices (e. g. timing and/or site of the application) can be used to impart selectivity. Glyphosate, a nonselective herbicide, is used prior to planting as a substitute for tillage, thereby taking advantage of its rapid and wide-spectrum weed killing activity and short lifetime in the soil.

A number of important classes of herbicides (e.g. the triazines, sulfonylureas, and imidazolinones) are more toxic to weeds than to specific crops. In these examples, selectivity results from a unique or enhanced metabolic detoxification of the herbicide by the crop plant but not by the weed. In other cases, herbicide selectivity results from the sequestering of the herbicide within an internal compartment of the crop plant. Alternatively, external barriers such as plant cuticles can prevent penetration of the herbicide. In some cases it has been possible to achieve selectivity by seed coat applications of a "safener," a second chemical that reduces the toxicity of the herbicide to the crop.

Genetic modification of crops to make them herbicide resistant could remove a major factor in determining the choice of herbicides available for use by farmers. It could allow for the wider use of more effective herbicides with broader weed-control spectrums. In addition, compounds that are effective at low application rates, have short lifetimes in the soil, and have more favorable toxicological properties might become more generally useful if the constraint of crop selectivity were removed. These possibilities would extend beyond major acreage crops to minor acreage crops that have not yet benefited from effective weed control compounds.

Genetic modification could also complement and enhance existing herbicide selectivity by increasing the margin of safety for selective compounds, particularly during periods of environmental stress when plant metabolism is reduced. It could also allow the grower to increase the application rates for selective herbicides, thus leading to improved and/or wider-spectrum weed control for these compounds. The introduction of herbicide resistance into crops could give the grower greater flexibility in choosing crops for rotations or double crop plantings. (Such choices are currently limited by the differential sensitivities of crops to particular herbicides.) Thus the combination of crop protection chemical technology and genetic technology will provide a new range of management options for more effective weed control.

PLANTS RESISTANT TO AMINO ACID BIOSYNTHESIS INHIBITORS

Sulfonylureas and Imidazolinones

MODE OF ACTION The sulfonylureas are a broad class of compounds, many of which have herbicidal activity (Table 1). The sulfonylurea herbicides are notable for their low use rates (as low as 2 grams per hectare) and their low toxicity to animals. Thousands of analogues of the sulfonylureas have been synthesized and screened to find compounds selectively active against weeds. A number of such herbicides have been identified and commercialized. Selective toxicity in all of these cases results from the metabolic detoxification of the herbicide by a particular crop species. For example, in wheat the compound chlorsulfuron is hydroxylated by a cytochrome P-450 enzyme and then glycosylated (116). In soybeans, conjugation with homoglutathione plays a major role in detoxification of chlorimuron ethyl, as is the case for the nonsulfonylurea herbicides acifluorfen and metribuzin (42, 43).

Selective sulfonylurea herbicide toxicity has also been achieved by genetic modification of crops. The first example of a sulfonylurea herbicide–resistant mutant plant was obtained by selection of tobacco cells in tissue culture and the subsequent regeneration of mutant plants from the cell lines. Resistance was semi-dominant and segregated as a single nuclear gene (16). It has also been possible to isolate resistant mutants of other crop plants (e.g. soybeans) by seed mutagenesis, but considerable effort and time have been required (101). A third approach has been to engineer sulfonylurea-resistant crops through plant transformation. For this effort, the gene(s) responsible for the resistance trait, either the native genes in the metabolic detoxification pathway or the mutant genes identified by selection, had to be isolated. The use of microbial species as models has played a central role in the identification and isolation of the necessary plant genes.

Studies on the mode of action of the sulfonylurea herbicide chlorsulfuron showed that inhibition of cell division in plant tissue was an early response to treatment (91, 92). This result, along with the ability to isolate resistant mutants in cultured plant cells, suggested that the herbicide antagonized a single basic cellular function and encouraged the use of microbial models to investigate herbicide action. Physiological studies in *Salmonella typhimurium* suggested that the target of the sulfonylurea herbicide sulfometuron methyl was the enzyme acetolactate synthase [ALS (E.C.4.1.3.18); also known as acetohydroxy acid synthase, AHAS], which is required for the synthesis of isoleucine, leucine, and valine (see Figure 1; 65). Multiple ALS isozymes exist in the enterobacteria *S. typhimurium* and *Escherichia coli,* and it was

shown that ALS II and ALS III, but not ALS I, are inhibited by sulfometuron methyl (66). In vitro analyses of ALS activity from yeast, pea, tobacco, and *Chlamydomonas* demonstrated that the eukaryotic enzymes are very sensitive to sulfometuron methyl (15, 37, 49, 93).

Proof that the sulfonylurea herbicides act by inhibition of ALS came from a combination of genetic and biochemical studies. Sulfonylurea-resistant mutants of *S. typhimurium, Saccharomyces cerevisiae, Nicotiana tabacum,* and *Arabidopsis thaliana* were isolated. Most of the mutants from each organism produced ALS activity insensitive to the herbicide, and the resistant enzyme activity cosegregated with cellular resistance in genetic crosses (15, 37, 51, 65). The bacterial and yeast mutations were mapped to the loci of the ALS structural genes *ilvG* and *ILV2,* respectively (37, 65). The identification of ALS as the target of the sulfonylurea herbicides provided at least a partial explanation of their low toxicity to animals: Animals lack this enzyme and must obtain the branched-chain amino acids from their diets.

ALS is also the target of two other structurally distinct classes of herbicides, the imidazolinones (Table 1; 83, 105) and the triazolopyrimidines or sulfonanilides (52, 63). Thus, ALS may be a particularly susceptible target for herbicides. It has been demonstrated that the toxicity of sulfometuron methyl to bacteria is enhanced by the accumulation of an ALS substrate, 2-ketobutyrate, which is itself toxic. It has therefore been suggested that the deficiency of branched-chain amino acids and the increase in concentration of the toxic intermediate combine to make ALS a particularly good target for herbicides (67). However, no evidence that 2-ketobutyrate is accumulated in and/or is toxic to plant cells has been reported.

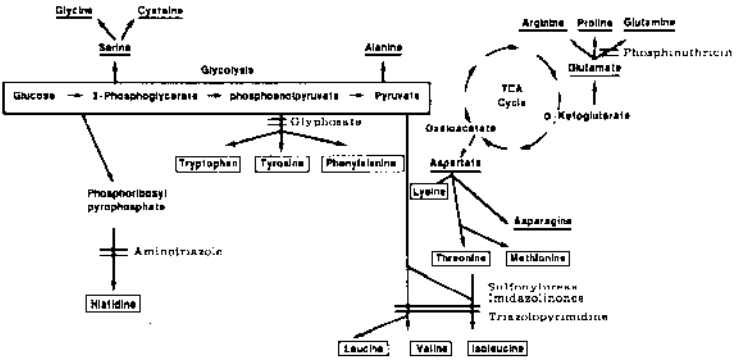


Figure 1 Herbicide targets in amino acid biosynthetic pathways. The figure shows the pathways for amino acid biosynthesis and the points in these pathways where herbicides or classes of herbicides can interrupt amino acid synthesis. Boxes indicate essential amino acids.

BIOCHEMICAL AND GENETIC CHARACTERIZATION OF ALS Genetic and biochemical studies have provided strong evidence that ALS enzymes from enteric bacteria are tetramers containing two subunits. ALS I purified from *E. coli* is composed of two large 60 kD and two small 9.5 kD subunits (34). Molecular cloning and DNA sequencing have provided a physical characterization of the *ilvBN* operon that encodes these subunits (44, 123). Similarly, ALS II has been purified from *S. typhimurium* and shown to be composed of two large 59 kD and two small 9.7 kD subunits. DNA sequence analysis of the cloned *E. coli ilvGMEDA* operon, along with amino acid sequence analysis of purified ALS II, has demonstrated that the *ilvG* and *ilvM* genes encode these subunits (68, 100). Genetic studies first suggested that ALS III was specified by two genes, designated *ilvI* and *ilvH* (26). DNA sequence analysis again provided physical evidence for the existence of the two genes, organized as an operon (110). Purification of ALS III provided confirmation of an analogous subunit structure for this isozyme (6). Although the early genetic studies on ALS III suggested a role for the small subunit in controlling the sensitivity of the enzyme to valine feedback inhibition, more recent work has indicated an important role for the small subunit in enzyme activity. Mutational inactivation of the small subunit genes resulted in a 20- to greater than 100-fold reduction in ALS activity (35, 72, 111).

Comparison of the inferred amino acid sequences of the bacterial ALS enzymes has indicated a common evolutionary origin. This similarity is most obvious among the large-subunit polypeptides, which share about 40% amino acid sequence identity concentrated in three regions of the protein (see Figure 2). Amino acid sequence conservation, although still evident, is less extensive between the small-subunit genes (44, 110, 123).

The molecular cloning and DNA sequence analysis of the yeast ALS gene, designated *ILV2*, permitted a comparison of the ALS amino acid sequence from a eukaryotic source with those from *E. coli* (37, 38). Similarity between the 687-amino-acid-long *ILV2*-encoded polypeptide and each of the *E. coli* large-subunit polypeptides was as extensive as that between the *E. coli* polypeptides (38). The most striking structural difference between the deduced yeast and bacterial proteins was the presence of an approximately 90-amino-acid-long amino terminal sequence extension on the former that was absent from the latter. Because sub-cellular fractionation experiments (98) had shown yeast ALS to be localized in mitochondria and because the amino acid sequence in the region has characteristics common to other known mitochondrial transit sequences (31), it was suggested that this region might include a mitochondrial transit sequence (38).

HERBICIDE RESISTANCE MUTATIONS Cloned yeast and bacterial ALS genes were used to investigate the molecular basis for resistance to the

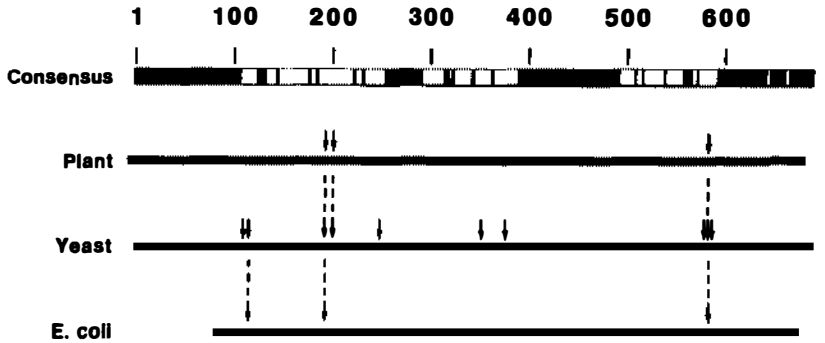


Figure 2 Comparison of ALS amino acid sequences. The top bar shows a schematic representation of homologies between *E. coli* ALS I, II, and III, yeast ALS, and ALS from the plants tobacco and *Arabidopsis*. The white boxes show stretches of amino acids that are conserved between all enzymes; the black boxes depict nonconserved regions. The arrows intersecting the horizontal lines show positions in these sequences where substitutions can produce a herbicide-insensitive enzyme. Dashed vertical lines indicate positions where such substitutions occur in more than one species.

sulfonylurea herbicides. Overexpression of the yeast ALS enzyme by about 4-fold occurred when the gene was present on a high-copy-number plasmid; this overexpression resulted in a 4–5-fold increase in the minimal concentration necessary to inhibit growth (37). An increase in the minimal inhibitory concentration of a sulfonylurea herbicide for *E. coli* was also observed when a functional *E. coli ilvG* gene was present on a high-copy-number plasmid (124). Mutations that resulted in the production of sulfonylurea-resistant ALS were isolated in the cloned yeast *ILV2* and *E. coli ilvG* genes by genetic selection. DNA sequencing showed that each mutant gene contained a single nucleotide change, resulting in a single amino acid substitution in the ALS protein. In yeast ALS, pro192 was substituted with ser, and in *E. coli* ALS II ala26 was changed to val (124).

Genetic engineering of yeast ALS was used to discover other mutations that resulted in sulfonylurea herbicide resistance. Spontaneous mutations in the yeast *ILV2* gene were isolated and characterized by DNA sequencing to determine the amino acid substitutions responsible for herbicide resistance (40). This analysis showed 24 different amino acid substitutions, at 10 different sites, ranging from the amino to the carboxy ends of the protein (Table 2, Figure 2).

The amino acid residues present in the wild-type yeast enzyme at these 10 sites are also present in the three wild-type *E. coli* ALS isozymes at most sites (40). One of the exceptions to this generalization occurs in *E. coli* ALS II, where a serine residue is present at the site analogous to that where the proline

Table 2 Herbicide resistant yeast ALS mutants

Wild type amino acid residue	Amino acid substitutions resulting in resistance
116G	S N
117A	P S T I L V N Q D E K R H W F Y M
192P	A S V Q E R W Y
200A	T V D E R W Y C
251K	P T N D E
354M	V K C
379D	G P S V N E W
583V	A N Y C
586W	G A S I L V N E K R H Y C
590F	G L N R C

to serine substitution had resulted in herbicide resistance in yeast. Since *E. coli* ALS II was known to be more resistant to the sulfonylurea herbicide sulfometuron methyl than were the yeast or plant enzymes (39), it was plausible that this serine residue contributed to the increased resistance. This hypothesis was tested by converting the serine codon in the ALS II gene of *E. coli* to a proline codon by site-directed mutagenesis; this change indeed increased the sensitivity of the enzyme to the herbicide. In another example, *E. coli* ALS I, which is much more resistant to the sulfonylurea herbicides than is ALS II, had a glutamine residue at one site analogous to that where substitutions for the wild-type tryptophan residue had resulted in herbicide-resistant yeast ALS, and a serine residue at a second site where substitutions for the wild-type alanine residue had resulted in herbicide resistance in both yeast ALS and *E. coli* ALS II. Conversion of the glutamine residue in *E. coli* ALS I to tryptophan also resulted in increased sensitivity to the herbicides (9). Thus these residues appear to contribute to inhibition by sulfonylureas in all ALS enzymes. The herbicide-resistant plant enzymes for which sequence information is available also have amino acid substitutions at these same sites (see below).

Site-directed mutagenesis was used to expand the spectrum of amino acid substitutions at the ten sites in yeast ALS (40). At some of these sites, such as ala117, pro192, and trp586, nearly any substitution for the wild-type amino acid resulted in a herbicide-resistant enzyme. At other sites, only a few substitutions had that result. A list of sulfonylurea herbicide–resistant yeast ALS mutants is shown in Table 2.

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cide-resistant transgenic plants faced a number of difficulties and uncertain-

ties. Two of these derived from the knowledge that plant ALS is localized in the chloroplast (58, 79) and that bacterial ALS is composed of two different subunits. Thus the generation of herbicide-resistant plants using bacterial genes might have required not only expression of the two protein subunits of the enzyme, but also their translocation into and assembly in plant chloroplasts. Since ALS had not been purified from yeast, neither the subunit structure of the enzyme nor the amino terminus of the mature protein present in the mitochondria was known, adding considerable uncertainty to any effort to express that enzyme in plants. For these reasons the isolation and use of plant ALS genes appeared to be a more attractive route for engineering sulfonylurea-resistant transgenic plants.

Even before the conservation of amino acid sequences between yeast and bacterial ALS enzymes had been discovered, hybridization between the yeast and *Salmonella ilvG* genes had been detected under low-stringency conditions (75). Together, these observations led to an attempt to detect ALS genes from other species, using heterologous DNA hybridization. A segment of the yeast ALS gene that spanned most of the coding region was used as a probe. Hybridization was detected between the yeast ALS gene and genomic DNA libraries from the cyanobacterium *Anabaena* 7120 and the higher plants *A. thaliana* and *N. tabacum;* ALS genes were isolated from all three species (76).

DNA sequence analysis of the *Arabidopsis* and *Nicotiana* ALS genes indicated that neither gene has introns, and that they code for proteins of 667 and 670 amino acids, respectively. The deduced ALS protein sequences are similar throughout most of their length; approximately 75% of the nucleotides and 85% of the encoded amino acids are identical (76). The 5' ends of the coding sequences, which are the only regions that are not highly conserved between the two plant ALS sequences, appear to encode chloroplast transit sequences. The nucleotide sequences of these regions are more similar than are the deduced amino acid sequences, suggesting that there are few constraints on the amino acid sequences in the transit peptides. Comparison of the deduced amino acid sequences of the plant ALS genes with those of the yeast and the three *E. coli* ALS genes showed that all share the same three conserved domains (Figure 2). At the ten sites where substitutions result in sulfonylurea-resistant yeast ALS, the amino acid residues present in the plant ALS enzymes are identical to those found in the wild-type yeast enzyme.

The number of ALS genes present in *N. tabacum* and *A. thaliana* was determined by Southern blot hybridization analyses. Homologous cloned ALS genes were used as probes. A single ALS gene hybridized to the probe in *Arabidopsis*, while two hybridized in *N. tabacum* (an allotetraploid) (76). The identification of two ALS genes in tobacco was consistent with genetic data,

which had indicated that tobacco mutations that confer herbicide resistance define two loci (15, 16). Southern blot analyses of DNA from several other crop species has indicated that many, such as corn and soybean, carry multiple ALS genes (B. J. Mazur, unpublished observations).

The cloned plant ALS genes were used as hybridization probes to isolate genes carrying ALS mutations from herbicide-resistant plants. In tobacco, the Hra line, which is mutated at the *SURB* locus and which is 1000-fold more resistant to sulfonylureas than are wild-type lines (17), was used as one source of a mutant ALS gene. A second tobacco line, C3, which carries a mutation at the *SURA* locus (16), was used as a source of a second mutant ALS gene. Four genes, representing all of the ALS loci from the two mutant lines, were isolated and sequenced. The molecular characterization of mutant and wild-type genes from each plant line permitted the assignment of the genes to the appropriate genetic locus and the determination of the amino acid substitutions in the mutant enzymes. The *SURB-Hra* gene, which was isolated by two successive rounds of genetic selection, contained two mutations, which resulted in pro196 to ala and trp573 to leu substitutions. The *SURA-C3* gene carried a single mutation that resulted in a pro196 to gln substitution (70). Similarly, a gene that conferred sulfonylurea herbicide resistance was isolated from *Arabidopsis* and sequenced; this gene carried a single mutation that resulted in a pro197 to ser substitution (50). Both the pro196 to gln mutation in the tobacco gene and the analogous pro197 to ser mutation in the *Arabidopsis* gene conferred selective resistance to sulfonylurea herbicides but not to imidazolinone herbicides. The double tobacco mutant that carried the pro196 to ala and trp573 to leu substitutions was cross-resistant to both classes of herbicides (70).

HERBICIDE-RESISTANT TRANSGENIC PLANTS The mutant plant ALS genes were introduced into *N. tabacum* by transformation and conferred useful levels of herbicide resistance in transgenic plants (50, 70). The tobacco *SURB-Hra* gene was also used to transform a number of heterologous species to sulfonylurea herbicide resistance at the cellular, and in some cases, whole plant level. These species include tomato, sugarbeet, oilseed rape, alfalfa, lettuce, and melon (9). In some of the heterologous transformants, such as tomato, expression of the resistant tobacco ALS gene was efficient, 20–60% of ALS activity being derived from the mutant gene (39, 77). In others, such as rape, only a low level of resistance was observed (9). Thus the effectiveness of heterologous genes must be evaluated on a case-by-case basis.

Additional herbicide-resistance mutations in plant ALS genes were generated by site-directed mutagenesis, based upon the mutations identified in the yeast ALS gene. The mutant genes were introduced into tobacco by transformation, and their ability to confer herbicide resistance was monitored

(48). In addition, a bacterial expression assay system for the *Arabidopsis* ALS gene was developed to permit the rapid isolation and characterization of new mutations. In this assay system, the plant gene, including its chloroplast transit sequence, was expressed in an *E. coli* auxotroph that produced no ALS. The wild-type *Arabidopsis* ALS gene promoted growth only in the absence of herbicides, while mutant *Arabidopsis* ALS genes promoted growth in both the presence and absence of herbicides. This selection thus allowed a facile assay for the efficacy of herbicide-resistance mutations in a plant ALS gene (107).

The *SURB-Hra* gene was introduced into a number of commercial lines of tobacco, and the transformed plants were tested to determine whether they could manifest useful levels of resistance in the field. Prior to field testing, the transformed plants were assayed for levels of sulfonylurea resistance by several methods. Resistance was measured by assaying leaf ALS activity in the presence of herbicide, by measuring secondary callus growth in the presence of increasing concentrations of herbicide, by monitoring the ability of progeny seeds to germinate and grow in the presence of increasing concentrations of herbicide, and by monitoring plant phytotoxicity after foliar spray applications of herbicides in greenhouse trials. The results of each of the tests were consistent but indicated the need for careful screening in order to identify those lines most suitable for crop breeding (39, 77, 106). For breeding purposes, a high level of herbicide resistance originating from a single genetic locus is preferred.

As a more critical measure of agronomically useful herbicide resistance, some of the tobacco transformants were treated with sulfonylurea herbicides in field tests and evaluated for phytotoxic symptoms. Foliar sprays were applied at rates corresponding to 0, 8, 16, and 32 grams of herbicide per hectare. Transformed plants showed no damage at the highest application rate tested, which was more than four times that of a typical field application rate. Wild-type plants showed damage at the 8g/hectare application rate (Figure 3; 77). Thus, expression of the *SURB-Hra* gene can provide an effective means of producing sulfonylurea herbicide–resistant crops.

ALTERNATIVE STRATEGIES FOR ACHIEVING HERBICIDE RESISTANCE The metabolic detoxification of particular sulfonylureas by specific crop species, which is the basis for the selectivity of commercial herbicides, could provide another approach for genetically engineering sulfonylurea resistant crops. Native genes responsible for the detoxification pathways could be isolated from tolerant crops and transferred to sensitive ones. Again a microbial model has proved useful. In *Streptomyces griseolus* sulfonylurea herbicides such as chlorsulfuron, sulfometuron methyl, and chlorimuron ethyl are metabolized by a three-step process requiring an NADP-dependent reductase,

Figure 3 Field trial of herbicide-resistant transgenic tobacco plants. A gene encoding a sulfonylurea herbicide–insensitive form of tobacco ALS was transferred to elite cultivars of tobacco. The resulting transgenic tobacco plants were tested for sulfonylurea resistance in a field trial. Transgenic plants *(left and right)* were healthy, while nontransformed plants *(center)* were severely stunted by the treatment.

an iron-sulfur protein, and either of two distinct, inducible cytochrome P-450 enzymes (88, 96). This bacterium could serve as a source for the isolation of the genes encoding the metabolically active proteins. These genes could be introduced into plants by transformation to confer herbicide resistance. Unfortunately, most of the same difficulties and uncertainties associated with the expression of microbial genes encoding herbicide-resistant ALS in plants also exist for the microbial detoxification genes. As an alternative, the bacterial genes might serve as probes for the isolation of plant genes encoding detoxifying enzymes, in an approach analogous to that used with ALS.

Because the most agronomically important crops were not amenable to transformation, efforts were mounted to generate resistance to the sulfonylureas and the other ALS inhibitors using alternative methods. Imidazolinone-resistant mutants of maize were isolated using genetic selection of cultured maize cells. Fertile plants exhibiting a greater than 100-fold increase in resistance to imidazolinones and cross-resistance to the sulfonylureas were regenerated from one line. Homozygous progeny showed more than a 300-fold increase in resistance to the herbicides. Resistance was inherited as a single dominant nuclear gene, and ALS activity from the mutants was resis-

tant to the herbicides in vitro (3, 4). This mutant, which did not appear to have any associated growth or yield defects, is being developed by Pioneer Hi-Bred International, Inc. Although a significant breeding program has been required to introduce the resistance trait into elite lines, it is likely to be the first ALS-targeted herbicide-resistant crop to be commercialized, with release anticipated in the early 1990s. Imidazolinone-resistant mutants of maize that show no cross-resistance to the sulfonylureas have also been isolated. In one case the mutant showed tolerance to a much lower level of herbicide. Tolerance was inherited as a recessive trait, and no change in ALS activity was observed (3).

Sulfonylurea herbicide–tolerant mutants of soybean have been obtained by genetic selection from mutagenized soybean seeds. The first mutants to be described showed only a 5–10 fold increase in tolerance, were recessive, and did not affect the sensitivity of ALS to inhibition by sulfonylureas (101). Recently, sulfonylurea-resistant mutant lines of soybean with significantly higher levels of tolerance have been obtained. These mutants have an altered ALS that shows reduced sensitivity to inhibition by sulfonylureas (S. A. Sebastian, personal communication). There have been no reports yet of mutants selected for resistance to the triazolopyrimidine class of inhibitors.

Glyphosate

MODE OF ACTION Glyphosate (Table 1) is an exceptionally reliable, phloem mobile, broad-spectrum herbicide with little residual soil activity. Because of these desirable properties, because it is toxic to all major crops, and because of its considerable commercial importance, extensive efforts have been aimed at the development of herbicide-resistant cultivars. A number of recent reviews have described this work in depth (21, 61, 89, 104).

The primary target of glyphosate is 5-enolpyruvyl-shikimate-3-phosphate synthase, or EPSPS, an enzyme in the aromatic amino biosynthetic pathway (see Figure 1). This pathway is found only in microbes and plants; glyphosate exhibits low acute toxicity to mammals. EPSPS was originally inferred to be the target of glyphosate action through identification of shikimic acid as an intermediate that accumulated following glyphosate treatment, through experiments showing that glyphosate activity could be suppressed by the addition of aromatic amino acids (57), and by biochemical studies of glyphosate inhibition of EPSPS (1, 55, 114). These findings were subsequently corroborated by the selection of glyphosate-resistant mutant strains of enteric bacteria. A resistant strain of *Salmonella* was identified following two cycles of chemical mutagenesis. The resistance mutation was shown to be in the *aroA* gene; the mutant gene was subsequently isolated, and was shown to confer resistance when transferred to *E. coli*. DNA sequencing indicated that two distinct types of mutations had led to the glyphosate-resistance phenotype.

The first round of mutagenesis had created a promoter mutation in the *aroA* gene, which conferred low levels of glyphosate tolerance by elevating the level of expression of the gene. The second cycle of mutagenesis had generated to a point mutation in the *aroA* structural gene, which caused a proline to serine substitution at residue 101 of the protein (20, 112). Overexpression of the *E. coli aroA* gene, as a consequence of its presence on a high-copy-number plasmid, could also confer glyphosate tolerance (95), providing additional evidence that EPSPS was the primary target of this compound.

HERBICIDE-RESISTANT TRANSGENIC PLANTS Glyphosate-tolerant transgenic plants were first generated by transferring the mutant *Salmonella aroA* gene, linked to either an octopine or mannopine synthase promoter for plant cell expression, to tobacco (19). The bacterial gene lacked a chloroplast transit sequence, and thus the herbicide-resistant EPSPS was expected to be localized in the cytoplasm; the plant EPSPS is predominantly localized in the chloroplast (28, 82). The transformed tobacco plants showed increased, but incomplete, tolerance to glyphosate. The same bacterial gene was also transferred to tomatoes. The gene again conferred glyphosate tolerance, but after foliar herbicide treatments the transformants were smaller than the unsprayed controls (41). Subsequent to these studies, mutant bacterial genes were fused to plant EPSPS chloroplast transit sequences (see below) and then transferred to plants. In one series of experiments, the petunia EPSPS gene transit sequence, along with the first 27 codons for the mature protein, was fused to a mutant *E. coli aroA* gene. The chimeric preprotein was imported into petunia chloroplasts and conferred glyphosate resistance in the transformed plants (29, 89).

Glyphosate resistance has also been imparted to plants through transformation with plant EPSPS genes. The plant genes were isolated by taking advantage of the finding that plant lines tolerant to glyphosate arose through overproduction of EPSPS (2, 85, 115). A glyphosate-tolerant petunia line was isolated by applying increasingly stringent stepwise selection conditions until a line was established in which EPSPS DNA, RNA, and protein levels were elevated approximately 20-fold (104, 115). This elevation in EPSPS-specific macromolecules facilitated the subsequent cloning of the EPSPS gene. EPSPS was purified from the line, and the N-terminal amino acid sequence of the protein was determined by microsequencing. Based on this sequence, three sets of potentially complementary oligonucleotide probes for the gene were synthesized and were used to screen messenger RNA populations from the amplified line. The set containing the oligonucleotide complementary to the gene was determined from Northern blot hybridizations, and was then used to identify a partial cDNA clone for EPSPS. The cDNA clone was in turn used

to identify a genomic DNA clone; a complete cDNA clone was subsequently constructed (104).

The petunia EPSPS gene was shown to be a nuclear gene that spans 9 kb of DNA and is interrupted by 7 introns. The mature protein was predicted to have a molecular mass of approximately 48 kD (45). A comparison of the inferred petunia, *Arabidopsis,* and tomato EPSPS proteins showed that they are highly conserved, except in the region of the chloroplast transit peptide (45, 62). The petunia gene codes for a 72-amino-acid chloroplast transit sequence, while the tomato chloroplast transit sequence is more than twice as long, with 148 codons.

The petunia EPSPS gene was used to produce glyphosate-resistant transgenic plants. The petunia cDNA clone was linked to a Cauliflower Mosaic Virus 35S promoter, in order to obtain a high level of expression of the gene in plants. Expression of the wild-type gene from this promoter resulted in a 20-fold increase in EPSPS activity in transgenic petunia plants. The plants tolerated applications of glyphosate approximately four times greater than that needed to kill nontransformed plants (103). In order to provide additional herbicide tolerance in the transgenic plants, particularly in the meristematic regions where glyphosate accumulates (80), site-specific mutations were introduced into the wild-type petunia EPSPS gene. All of the substituted EPSPS proteins had reduced catalytic efficiencies (61, 89). The modified genes were again coupled to the Cauliflower Mosaic Virus 35S promoter, and were able to confer higher levels of resistance in transgenic plants. In a parallel series of experiments, mutated bacterial EPSPS genes were fused to plant EPSPS chloroplast transit sequences, and the chimeric genes introduced into plant cells (see above). These constructions also conferred herbicide resistance (61). Using such herbicide-resistance constructs, a wide variety of glyphosate-resistant transgenic plant species have been created. Some of these plants, including tomato and oilseed rape, have been tested in field trials during 1987 and 1988.

ALTERNATIVE STRATEGIES FOR GLYPHOSATE TOLERANCE *Pseudomonas and Arthrobacter* species capable of growing on glyphosate as a sole carbon source have been identified (59, 90). These strains metabolize glyphosate to phosphate, glycine, and a one-carbon unit (56, 60, 90). In contrast, through soil organism metabolism, glyphosate is primarily degraded to aminomethylphosphonic acid (86, 97, 109). The bacterial genes that carry out these metabolic degradations could theoretically be isolated and expressed in plants, to produce glyphosate-tolerant plants. No glyphosate-tolerant plants have yet been reported in which expression of cloned genes enables metabolism of glyphosate to nontoxic products.

Phosphinothricin

MODE OF ACTION Phosphinothricin (PPT) is an analogue of glutamine that inhibits the amino acid biosynthetic enzyme glutamine synthetase (GS) of bacteria and plants (see Figure 1) (8, 69). Bialaphos is a tripeptide precursor of PPT produced by some strains of *Streptomyces,* in which two alanine residues are linked to the PPT moiety; the active PPT moiety is released intracellularly by peptidase activity. Both PPT and bialaphos are marketed as broad-spectrum contact herbicides; they are not phloem mobile. Inhibition of GS by these compounds causes a rapid buildup of intracellular ammonia levels and an associated disruption of chloroplast structure, resulting in the inhibition of photosynthesis and plant cell death (117). Even though PPT inhibits GS from bacteria, plants, and mammals, its inability to cross the blood-brain barrier and its rapid clearance by the kidneys are the apparent reasons for its nontoxicity to mammals.

HERBICIDE-RESISTANT TRANSGENIC PLANTS Several approaches have been used to produce plants tolerant to these herbicides. An alfalfa cell line tolerant to PPT was isolated following selection and was shown to produce elevated levels of GS (30). This line was used to facilitate the purification of GS. The amino acid sequences of two internal GS peptides were subsequently used to confirm the identification of an alfalfa cDNA clone (30), which had been isolated through hybridization with a *Phaseolus* GS cDNA clone (22). The corresponding genomic DNA clone for alfalfa GS was shown to be 4 kb long and to contain 11 introns. The encoded protein was predicted to be 356 amino acids long and to share several regions of sequence similarity with the *Anabaena* and Chinese hamster enzymes. The original tolerant line was demonstrated to have a 35-kb segment of DNA, carrying the coding region for GS, which had been amplified approximately 10-fold (120). In order to produce herbicide-resistant transgenic plants, the alfalfa GS cDNA clone was linked to the Cauliflower Mosaic Virus 35S promoter and introduced into tobacco plants via transformation. The resulting high level of expression of the alfalfa GS gene in the transgenic tobacco plants conferred a low level of tolerance to PPT (32).

In order to identify herbicide-resistance mutations that could occur in plant GS genes, and which might confer increased levels of tolerance to PPT, a bacterial expression assay system was developed. An alfalfa GS cDNA clone was linked to a bacterial promoter and transferred to a strain of *E. coli* devoid of GS activity; thus bacterial growth was dependent on the functional expression of the plant gene (24). Mutations that allowed bacterial growth in the presence of PPT were then selected. Such mutations, however, resulted from overexpression of the GS gene, as a consequence of gene amplification or

promoter mutations. This problem was circumvented by fusing the 5' end of the plant GS gene to the 3' end of the *E. coli lacZ* gene, thus allowing a β-galactosidase assay to be used to screen for GS overproduction. Several point mutations in the alfalfa GS gene were identified by this method. DNA sequencing of these mutations predicted substitutions of serine, cysteine, or arginine for gly245 and lysine for arg332 (H. M. Goodman, unpublished).

A mutant alfalfa GS gene was then transferred to tobacco cells, and plants were regenerated. Although the mutant GS gene protected plants when herbicide was taken up through the roots, it did not protect against foliar applications of herbicide (H. M. Goodman, unpublished). One possible explanation for this finding derives from the existence of multiple nuclear GS genes in plants (118). In pea, for example, one GS gene encodes an isozyme found in the nodules, a second encodes a GS isozyme found in chloroplasts, and two others encode cytoplasmic forms of GS (119). Thus resistance mutations may have to be introduced into several GS genes to confer whole-plant resistance to this herbicide.

An alternative approach to achieving PPT resistance has been more successful. In this case, resistance was introduced into plants via a gene that produces a detoxifying enzyme. The gene, designated *bar*, is found in strains of *Streptomyces* that produce bialaphos, the tri-peptide precursor of PPT. The *bar* gene product protects these strains from the action of their own antibiotic by metabolizing PPT to an inactive, acetylated derivative (121). The *bar* gene was isolated from *Shygroscopicus* (84), placed under the control of the Cauliflower Mosaic Virus 35S promoter, and introduced into tobacco, potato, and tomato (25). Expression of the gene produced transferase levels that varied from 0.001% to 0.1% of the soluble protein in transgenic plants; protection against PPT occurred even in the plants producing the lowest levels of transferase (71). Field trials of transgenic tobacco, tomatoes, potatoes, oilseed rape, and poplars resistant to PPT have been or will be conducted (J. Leemans, personal communication).

PLANTS RESISTANT TO PHOTOSYNTHESIS INHIBITORS

Atrazine

MODE OF ACTION The s-triazines (e.g. atrazine and simazine) and the ureas (e.g. diuron) (Table 1) are herbicidal because they inhibit photosynthesis. Atrazine was discovered in the 1950s and has become an important herbicide for corn and sorghum. These crops, in contrast to most other crops and weeds, have isozymes of glutathione-s-transferase that rapidly detoxify atrazine by conjugation. Because atrazine is slowly metabolized to inactive forms in the

soil, residues from one growing season to the next limit the grower's opportunity to rotate atrazine-treated corn and sorghum with other crops such as soybeans or small grains.

The extensive agricultural use of atrazine has provided a strong genetic selection for the emergence of resistant weeds. The first atrazine-resistant mutant weed was reported in 1970 (98). About 38 resistant weed species have now been identified in Canada, Europe, and the United States (47). Atrazine resistance is maternally inherited, indicating that the gene responsible is located in the plastid or mitochondrion (108). Considerable advantage has been taken of the resistant weeds in research on the atrazine target and on the photosystem II reaction center. The protein site of action of atrazine and related herbicides was identified by a combination of physiological, biochemical, and genetic studies. Early research indicated that these herbicides were inhibitors of photosynthesis. Binding studies showed that atrazine displaced a plastoquinone from its binding site on a membrane protein in the photosystem II complex of chloroplasts. This protein has been variously designated D1, the 32-kD membrane protein, the herbicide-binding protein, and the Q_β protein (73).

The Q_β protein has been extensively studied in several plants. It undergoes a more rapid turnover than other chloroplast proteins (74, 94), and its degradation and synthesis are light regulated (87). The protein, encoded by the chloroplast gene *psbA* (12), is synthesized as a 34-kD precursor (33, 46, 94) and is posttranslationally processed and assembled into photosystem II complexes (14). The *psbA* gene has been cloned and sequenced from several higher plants and photosynthetic microorganisms. The deduced amino acid sequences indicate that the Q_β protein is a highly conserved, hydrophobic protein of about 350 amino acids (36).

The herbicide-resistant weeds, as well as resistant mutants selected in several microorganisms, such as *Chlamydomonas, Euglena,* and the cyanobacterium *Anacystis,* have provided a source of genes for studies of the molecular genetic basis for resistance. More than a dozen mutant *psbA* genes have been isolated and sequenced; at least five sites where amino acid substitutions result in herbicide resistance have been identified (54). More than two thirds of the mutations result in a substitution of ser264 with gly or ala. A list of herbicide-resistance mutations identified in *psbA* genes is given in Table 3. Crystallographic studies on the photosynthetic reaction center of purple bacteria (27), as well as correlations between the structural organization and herbicide binding sites of bacteria and higher-plant photosystems II, have led to a model for the topology of the Q_β protein in the thylakoid membrane and a proposed herbicide binding niche (122).

HERBICIDE-RESISTANT PLANTS Atrazine-resistant weeds have been used to generate resistant crops by various means. The resistance trait has been

Table 3 Mutations in Q_B conferring herbicide resistance[1]

Amino acid change	Species	Relative resistance			
		Atrazine	Metribuzin	Diuron	Bromacil
219 Val → Ile	C. reinhardtii	2	15	1	1
251 Ala → Val	C. reinhardtii	25	1000	5	
255 Phe → Tyr	C. reinhardtii	15		0.5	1
264 Ser → Gly	A. hybridus	1000	200	1	
264 Ser → Gly	S. nigrum	1000		1	20
264 Ser → Gly	B. campestris	600	50	1	
264 Ser → Gly	P. paradoxa	>120	23	1.3	62
264 Ser → Ala	C. reinhardtii	100		10	
264 Ser → Ala	E. gracilis				
264 Ser → Ala	A. nidulans	17	5000	150	300
275 Leu → Phe	C. reinhardtii			5	4
264 Ser → Ala 255 Phe → Tyr	A. nidulans	360	200	300	330
264 Ser → Ala 255 Phe → Leu	A. nidulans	2.5	175	2650	35

[1] Taken from reference 54

transferred by sexual crosses from the weed *Brassica campestris* into several *Brassica* crops of commercial importance, such as oil seed rape, rutabaga, and Chinese cabbage (11). This work led to the release of the atrazine-resistant Canola cultivar "Triton," which was planted on about 250,000 acres in Canada in 1986, despite a yield penalty of about 20% associated with the resistant cytoplasm. In another such example, resistance from the weed *Setaria viridis* was crossed into the crop *Setaria italica* (23). Further such sexual crosses will be limited, however, because only a few resistant weeds are sexually compatible with crop plants. Protoplast fusion has also been used to transfer atrazine resistance between *Solanum* lines (5). In *Brassica*, transfer of atrazine-resistant cytoplasm into cytoplasmic male sterile lines by protoplast fusion was carried out to aid hybrid seed production (7, 10).

Even with this wealth of information and the availability of a number of resistance genes, genetic engineering of triazine herbicide–resistant crop

plants via transformation has been difficult. A major problem has been the inability to introduce genes into chloroplasts reproducibly. To bypass this technical problem, transgenic tobacco plants were produced following nuclear transformation with a mutant *psbA* gene. To accomplish this, a chimeric gene was constructed in which a nuclear promoter and a chloroplast transit peptide-encoding sequence were attached to a mutant *psbA* structural gene. The transgenic plants showed an increased tolerance for atrazine (18).

As an alternative, engineering atrazine resistance through the introduction of genes encoding detoxifying enzymes has been attempted but has also proven difficult. Genes encoding atrazine and alachlor detoxifying glutathione-S-transferases (GSTI and GSTIII) have been isolated from maize (81, 103). Preliminary studies indicating some increased tolerance to atrazine of transgenic tobacco plants expressing an atrazine-metabolizing GST gene have been reported (53).

Bromoxynil

Bromoxynil is a benzonitrile compound that inhibits photosynthetic electron transport. This broad-leaf plant herbicide is used for weed control in cereal crops (Table 1). In order to engineer crops resistant to the effects of this herbicide, a search was initiated for bacteria able to metabolically detoxify the compound. A strain of the soil bacterium *Klebsiella ozaenae,* which could use bromoxynil as its sole nitrogen source, was isolated. This strain produces a bromoxynil-specific nitrilase protein that detoxifies the compound by conversion to 3,5-di-bromo 4-hydroxybenzoic acid, in a single step (78). The *bxn* gene that encodes this enzyme was shown to be plasmid borne and was subsequently cloned (113). The gene has been placed under the control of plant promoters and has been transferred to tobacco and tomato plants; it confers resistance to bromoxynil herbicide treatments in these plants (D. M. Stalker, personal communication).

CONCLUSION

In the last decade major research efforts on the genetic engineering of plants have been initiated in academic institutions and government agencies. In the private sector, a number of biotechnology companies devoted to this endeavor have been established; some have since failed or been partially or completely subsumed by larger concerns. Virtually all of the major agrichemical companies have established research and development programs in this area.

The field's early dreams, (e.g. of creating crops that could fix their own nitrogen) have been joined by more technically attainable goals. The generation of herbicide-resistant crops has provided a focal point for research, in part because it appears readily achievable. The targets of most herbicides are

known, and many herbicide-resistant plants have been identified, either as weeds or as mutants selected in the laboratory. Furthermore, herbicide resistance clearly represents a valuable trait. From the standpoint of basic research, the availability of genetic determinants for herbicide resistance can provide a plant analog to the antibiotic-resistance genes that have proven so important for bacterial research. In agriculture, herbicide-resistant crops could lead to more cost effective weed control and could permit the wider use of more environmentally desirable herbicides.

Much of the work on herbicide resistance has centered on the isolation of the genes that could impart the herbicide-resistance trait and on the transfer of these genes into plants. The motivation for this work has come from the added flexibility and increased speed that gene transfer can provide. For example, gene transfer can allow another gene, which confers resistance to a second herbicide, to be linked on the introduced DNA. This second resistance trait could be useful either to prevent herbicide-resistant weeds from arising following prolonged use of a particular herbicide, or to allow use of multiple herbicides with differing attributes in order to achieve the broadest possible weed control. Gene transfer can also allow herbicide resistance to be introduced simultaneously into multiple crop species and into multiple cultivars of each of those species. Finally, because herbicide resistance is a selectable trait, genes whose phenotype cannot be readily scored can also be transferred if linked to the resistance trait. Examples of such genes would include disease resistance and nutritional quality traits.

What has been achieved so far? The first example of a herbicide-resistant crop, atrazine-resistant Canola, derived from crossing a resistant weed with a crop, has been released for use. Although there is a yield penalty associated with the use of this cultivar, it has gained acceptance because of the advantages it provides for weed control. Imidazolinone-resistant corn, selected from corn cells grown in tissue culture, is in the breeding and evaluation stage, with release of cultivars expected in the early 1990s. A sulfonylurea-resistant soybean line has been selected from mutagenized seed and is being introduced into soybean cultivars by standard crop breeding practices. Many examples of transgenic herbicide-resistant crops have been reported and are in various stages of evaluation and development. Not surprisingly, this approach has turned out to be a more complex undertaking than originally envisioned. For example, it has been difficult to obtain adequate levels of glyphosate resistance in some crops, using either mutant bacterial or plant EPSPS genes. Similarly, field-application-rate levels of resistance to phosphinothricin based on mutant plant GS genes have not yet been reported. It is encouraging that useful levels of sulfonylurea herbicide resistance have been obtained in transgenic plants using mutant plant ALS genes, demonstrating that a herbicide target-based engineering approach can work. Finally, resistance resulting

from expression of herbicide detoxifying enzymes has been accomplished for phosphinothricin and bromoxynil. The levels of phosphinothricin resistance achieved through expression of the *bar* gene from *Streptomyces* in plants are suitable for agricultural use.

In addition to these practical achievements, much has been learned as a result of the efforts to engineer herbicide-resistant crops. Considerable basic biological knowledge has been generated, along with a tremendous increase in the development of the technologies necessary for sophisticated genetic manipulation of plants. Much progress has been made in the identification of DNA sequences that control the expression of genes in plant cells. This information derives from efforts to express bacterial genes that encode antibiotic and herbicide-resistance determinants, and from investigations of the expression of plant genes that encode herbicide-resistance determinants. Similarly, an increased understanding of the signals that control targeting of proteins to organelles has resulted from investigations employing herbicide-resistance genes. The study of mutant herbicide target proteins has also provided insight into protein-ligand interactions, enzyme functions, and the photosystem II reaction center. New information has been obtained in the areas of plant physiology and metabolism from investigations of the mode of action of herbicides and of plant detoxification pathways. Studies on herbicide-resistant mutants have demonstrated several instances of plant gene amplification, illuminating the dynamic nature of the plant genome. On the other hand, genes introduced into plants by transformation, inserted at many different and apparently random sites, exhibit stable inheritance.

Methods for propagating plant cells in culture and for regenerating whole, fertile plants from cells have been improved, modified, and extended to an ever-increasing number of species (41a). Among the plant transformation techniques that have been developed are *Agrobacterium*-mediated DNA transfer to plant protoplasts (co-cultivation) or to leaf disks; direct DNA uptake into protoplasts, with or without electroporation; and DNA injection into plant cells via micropipet or particle gun. Vectors of increasing utility have been constructed for the *Agrobacterium*-mediated transformation method, which remains the most reliable and widely used procedure for several plant species. It seems certain that transformation methods for all the major crop species will soon be available.

Along with these technical achievements, the genetic engineering of herbicide-resistant crops has provided a link between academic and industrial research efforts that should benefit both. It has also provided a test case for government regulatory agencies to use in the development of public policy. The agricultural biotechnology industry has used herbicide-resistant crops as an example to refine the evaluation process for product development. Thus, genetic engineering of herbicide-resistant plants has been not merely an end

but also a means for exploring the potential of recombinant DNA technology in agriculture. The rapid progress already made will spur further research, innovation, and the development of commercial products.

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