HERBICIDE-RESISTANT CROPS

Agricultural, Environmental, Economic, Regulatory, and Technical Aspects

Edited by

Stephen O. Duke

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Chapter 10

GENETIC ENGINEERING OF CROPS FOR TOLERANCE TO 2,4-D

Danny Llewellyn
David Last

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1. PHENOXYACETIC ACID HERBICIDES

2,4-Dichlorophenoxyacetic acid (2,4-D) is a member of a class of herbicides with activity against many dicotyledonous weeds. Although it is an older-generation herbicide, introduced in the 1940s, it is still in general use in many countries. The chemical structure of 2,4-D and some of its more common relatives such as MCPA (2-methyl-4-chlorophenoxyacetic acid) are shown in Figure 1. The group includes butyric acid derivates such as 2,4-DB that are metabolized to active 2,4-D by ß-oxidation, giving them a degree of selectivity different from that of 2,4-D. Because many legumes lack the ß-oxidation activity, 2,4-DB can be used for weed control on legume-containing pastures or crops. The most notable member of the group is 2,4,5-T, which, as a mixture with 2,4-D, formed the notorious "Agent Orange" herbicide used for massive forest defoliation during the Vietnam War. The phenoxy herbicides such as 2,4-D are active as plant growth regulators or hormones with auxin activity. They are absorbed through the leaves and are translocated initially for short distances by

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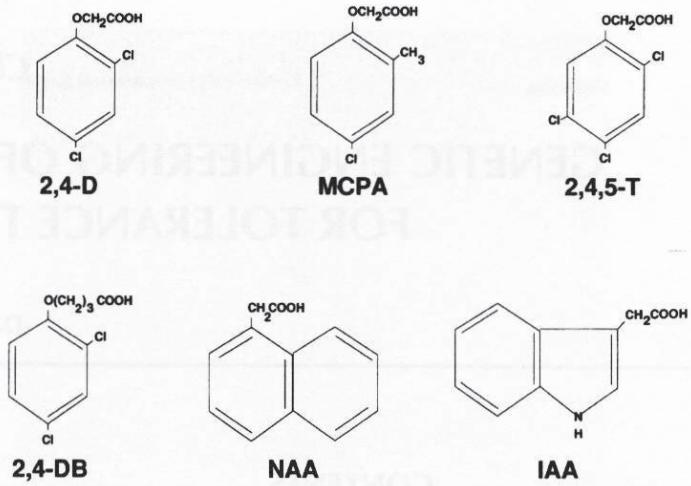

FIGURE 1 Chemical structures of phenoxyacetic acid herbicides and some natural plant auxins. 2,4-D, 2,4-dichlorophenoxyacetic acid; MCPA, 2-methyl,4-chlorophenoxyacetic acid; 2,4,5,-T, 2,4,5,-trichlorophenoxyacetic acid; 2,4-DB, 4-(2,4-dichlorophenoxy)butyric acid; IAA, indole-3-acetic acid; NAA, 1-naphthaleneacetic acid.

intercellular movement to the phloem and then over long distances in the phloem to the shoot meristems. Phloem-translocated herbicides such as the growth regulators move through the sieve tube system similar to sugars, from a source (region of sugar synthesis) to a sink (region of sugar utilization) (Singh and Muller 1979), although the exact mechanisms are still poorly understood. Stimulation of sink activity has been observed with 2,4-D (Kirkwood 1979), and this presumably potentiates its herbicidal activity. At the meristem 2,4-D and other phenoxy herbicides interfere with cell development, presumably through saturation effects arising from mimicry of auxin action on cell elongation (Dodge 1989). Characteristic damage to plants by low levels of 2,4-D is severe twisting and spindly growth of the vegetative and floral tips and organs (Figure 2), followed by the death of the meristems and eventually the plant.

Most commercial formulations contain salts, amine salts, or esters of the phenoxyacetic herbicides rather than the free acids. The esters are believed to be active only after hydrolysis to the free acid in the plant (Loos 1969). En route to the site of action the phenoxy herbicides are also exposed to the potential for modification to less active metabolites or in some cases to the active herbicide, e.g., 2,4-DB. Detoxification of the phenoxy herbicides may play an important part in the selectivity of these chemicals for different plant species. 2,4-D, for example, can undergo a variety of chemical modifications in insensitive plants (reviewed by Pillmoor and Gaunt 1981 and Loos 1969), including side chain degradation, side chain elongation, ring hydroxylation, and conjugation with proteins, amino acids, and glucose (Ashton and Crafts 1981).

Monocotyledonous plants are considerably less sensitive to phenoxyacetic herbicides and hence these herbicides are of particular use for broadleaf weed control in monocot crops such as cereals and sugarcane. 2,4-D is an important auxin in the tissue culture of cereals and is an active growth regulator for these species. It is not surprising, therefore, that cereals are sensitive to 2,4-D at particular developmental stages and can be seriously affected if this herbicide is used inappropriately (Evans 1968). However, the major scope for the production of crops resistant to 2,4-D is among the dicotyledonous crops that are sensitive to low concentrations of phenoxyacetic acid herbicides, particularly in multicropping systems where they may be exposed inadvertently to 2,4-D as spray drift or from residues in the soil or irrigation water. Among the dicot crops, the main focus of genetic engineering research worldwide has been into 2,4-D-tolerant cotton, a crop of considerable economic value that is extremely sensitive to 2,4-D.

FIGURE 2 2,4-D spray drift damage on cotton. Note the twisted distorted leaves and bracts and dead growing points.

2. CURRENT 2,4-D USAGE PATTERNS IN COTTON CROPPING

Weed control remains an important component of cotton production, primarily because of the noncompetitive nature of cotton seedlings (Keeley et al. 1986). Weeds can reduce cotton yields by direct competition for light, nutrients, and water, but can also affect quality parameters through contamination of the harvested lint. Weed spectrum will be determined by geographical location and weed management practices, but the problems are likely to be similar in all highly mechanized production systems such as those found in the U.S. and Australia. Charles (1991) surveyed the weed species and herbicide usage patterns on Australian cotton farms and found that most of the serious weeds were dicotyledonous. Generally, these were weeds of pastures and are either native or naturalized species. *Xanthium occidentale* (Noogoora burr), *X. spinosum* (Bathurst burr), and *Datura* spp. (thornapples) are large plants that are effective competitors with cotton and have been a long-term problem in cotton production. *Cyperus* spp. are perennial grasses adapted to moist soils and can be a problem in fields and drainage channels. Like the dicot weeds *Haloragis glauca* and *Polymeria longifolia,* these grasses have rhizomatous roots and are resistant to cultivation as a form of control. All of these species, except *Cyperus,* are sensitive to 2,4-D, but cannot be controlled effectively with this herbicide, except in fallow fields, because of the serious risk of damaging the cotton crop. 2,4-D is used on about one third of the cotton farms from ground rigs at any time after defoliation through to sowing the next season, but its popularity is reduced by fears of damage that could result from residues in the soil (Charles 1991). Current weed control programs rely heavily

on preemergence cultivation and use of nonselective herbicides such as glyphosate before planting, residual herbicides such as trifluralin and atrazine, and hand removal of weeds from postemergence cotton. 2,4-D is more commonly used on cereal crops (oats, barley, sorghum, and maize) in the same valleys in which cotton is grown or on fallow fields in wheat/fallow/cotton rotations to control broadleaf summer weeds. The majority of spraying of 2,4-D on cereals is done from the air, so spray drift, even on relatively calm days, away from the cereals into the cotton growing areas is almost inevitable. There are also instances in which 2,4-D residues are not removed from the tanks and have caused serious damage to cotton in subsequent spraying of an unrelated herbicide, miticide, or insecticide. In recent years there have been some isolated incidences of 2,4-D damage appearing in cotton as a result of its use on fallows. Such damage occurs when weather conditions do not favor growth of 2,4-D-degrading soil microbes, such as when it is unusually cold or dry (Hammond 1992). 2,4-D tolerant cotton would be useful both in the prevention of spray drift damage and perhaps for the control of some of the dicotyledonous weeds that currently trouble the industry by using this relatively cheap compound as an effective postemergence herbicide.

3. GENETIC ENGINEERING OPTIONS FOR 2,4-D RESISTANCE

Genetically engineered herbicide resistances are no longer new. Effective resistances have been developed to a variety of potent herbicides such as glyphosate and the sulfonylureas (Comai et al. 1985, Shah et al. 1986, Haughn et al. 1988). In these cases the molecular target of the herbicide was well characterized and resistances were developed through the manipulation or replacement of the target gene. Comai et al. selected a strain of *Salmonella typhimurium* that was resistant to glyphosate because of a mutation in the gene for 5-enolpyruvylshikimate 3-phosphate synthase (EPSP synthase), the target enzyme for glyphosate. When expressed in plants, the mutant enzyme could substitute for the endogenous plant enzyme that had been inactivated by the herbicide. Shah et al. used a different approach to achieve the same result. They selected a petunia cell line that was resistant to glyphosate through the overproduction of the EPSP synthase enzyme. This petunia gene was cloned and then reintroduced into petunia with a new, much stronger promoter. The new chimeric gene resulted in the over-production of the enzyme, and hence in resistance at a whole plant level. Haughn et al. achieved a similar result with an acetolactate synthase gene from *Arabidopsis,* which confers sulfonylurea herbicide resistance on transgenic tobacco plants. While all of these resistance genes are certainly effective, they may have some deleterious effects, especially in the absence of the herbicide.

Principles similar to those described above cannot be applied to developing a resistance gene against 2,4-D because its primary target is still unknown. 2,4-D is an analog of the plant hormone group known as auxins and at low levels is often used as a substitute for natural auxins in plant tissue culture systems. Some of the symptoms of 2,4-D damage to cotton such as twisting of shoots are suggestive of a hormone-induced mechanism such as perturbed rates of cell division. However, it is likely that there are other, more drastic and undetermined phytotoxic effects.

A second approach used to generate herbicide resistant plants is the engineering of detoxification pathways in susceptible species. A number of herbicides are toxic to some plant species but not others. The difference in sensitivity is often not associated with a difference in susceptibility of the molecular target, but to an ability of the resistant plant to detoxify or break down the herbicide before it has a chance to inactivate the susceptible target gene product. Two examples now exist in which a microbial gene encoding an enzyme that detoxifies a herbicide was modified for expression in plants and introduced into a model species by transformation (De Block et al. 1987, Stalker et al. 1988). The novel detoxification enzymes were expressed and conferred resistance to relatively high levels of phosphinothricin (Basta® or glufosinate) and 3,5-dibromo-4-hydroxybenzonitrile (bromoxynil), respectively. These artificial systems mimic detoxification systems found in plants and are likely to place the least genetic, physiological, or yield burden on the plant, both in the presence or absence of the herbicide. Furthermore, the mode of action of the herbicide need not be

known, provided that the appropriate detoxification genes can be found. This second strategy was the most promising for 2,4-D because a variety of microorganisms are known that naturally degrade the herbicide in the environment.

4. A MICROBIAL SOURCE OF 2,4-D DEGRADATION GENES

2,4-D has a relatively short residual life in soils, decreasing by about one half every 14 d or so (Noble and Hargreaves 1992). Other phenoxyacetic acid herbicides such as MCPA and 2,4,5-T break down much more slowly in the soil and can cause serious residual problems (Loos 1969). The breakdown of phenoxyacetic acid herbicides in the soil is performed by a variety of microorganisms including bacteria, yeasts, and fungi from several taxonomic groups. Loos (1969) lists many of these and the particular herbicides that can be used as substrates: a variety of *Pseudomonas* spp., *Achromobacter*, *Flavobacterium*, *Corynebacterium*, and *Arthrobacter* spp. and some Actinomycetes, such as *Nocardia* spp. and *Streptomyces viridochromogenes*, which will all degrade 2,4-D as a carbon source after appropriate enrichment of this substrate. The most well-characterized organisms are strains of *Alcaligenes eutrophus*, which are relatively simple Gram-negative rod bacteria found in most aerated soils. Several different strains of *A. eutrophus* were isolated from soils in fields commonly sprayed with 2,4-D and all are able to grow on synthetic media with 2,4-D as the sole source of carbon (Don and Pemberton 1981) (Figure 3). The common denominator between all the strains is a large (75 kb) plasmid that was shown to encode many of the enzymes necessary for the breakdown of 2,4-D. The overall pathway of 2,4-D degradation (Don et al. 1985) in *A. eutrophus* is shown in Figure 4.

It may be considered impractical to engineer all six *tfd* genes to function in plants, but data on the phytotoxicity of the first intermediate in the pathway, 2,4-dichlorophenol (DCP) suggested that only the first gene in the pathway may be necessary to confer a useful degree of tolerance on transgenic crop plants. Perkins et al. (1987) showed that DCP was toxic to cell suspension cultures of *Nicotiana tabacum* with a 50% inhibition at 10 mg/l and inhibited leaf and root development of tobacco seedlings at the same concentration. 2,4-D at 1 mg/l blocked all plant development. Streber and Willmitzer (1989) also tested the toxicity of DCP to tobacco callus and showed that DCP at 10 mg/l caused no obvious toxic effects, while 2,4-D at 2 mg/l severely reduced growth rates and turned the callus brown. Seed germination studies carried out by Llewellyn et al. (1990) in the presence of 2,4-D or DCP indicated that, in tobacco at least, DCP was some 50- to 100-fold less phytotoxic than 2,4-D. These results were encouraging with respect to expressing just the first step in the pathway for protection against 2,4-D damage.

Streber et al. (1987) were able to clone the *tfdA* gene encoding the first enzymatic step of 2,4-D degradation by Tn5 mutagenesis of the degradative plasmid of *A. eutrophus*. From >1000 kanamycin-resistant clones that had Tn5 insertions, two were identified that lacked the ability to cleave the acetate side chain of 2,4-D. A large *Hin*dIII fragment containing the wild-type plasmid DNA in the region of the tagged *tfdA* gene both complemented the mutations and allowed another strain of *A. eutrophus* (JMP222) to grow on 2,4-D analogs such as phenoxyacetic acid. JMP222 is able to grow on phenol as a carbon source by using chromosomal genes for a meta-cleavage pathway, but cannot normally grow on phenoxyacetate. The *tfdA* gene product is able to accept different substituted and unsubstituted phenoxyacetates as a substrate and can cleave phenoxyacetate to phenol. This assay was used to localize the gene by successive deletions of the large fragment down to 2 kb that could be sequenced. The *tfdA* gene was originally thought to encode a 2,4-D monooxygenase but subsequent biochemical analysis has indicated that the gene product is a ferrous iron dependent dioxygenase that uses α-ketoglutarate as a cosubstrate (Fukomori and Hausinger 1993). The α-ketoglutarate is converted to succinate and carbon dioxide concomitant with the conversion of 2,4-D to DCP and glyoxylate.

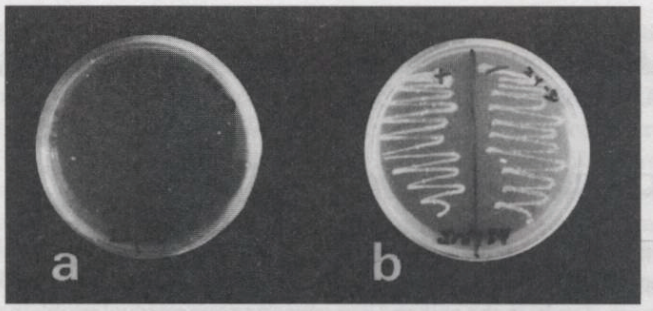

FIGURE 3 *Alcaligenes eutrophus* utilization of 2,4-D as a carbon source. Bacterial plates contain minimal medium supplemented with 125 µg/ml 2,4-D sodium salt as sole source of carbon. (a) JMP228R, a cured strain of *A. eutrophus* lacking the 2,4-D degradative plasmid is unable to grow on 2,4-D. (b) JMP134, a strain of *A. eutrophus* which has the degradative plasmid pJP4, grows on 2,4-D in the absence of any other carbon source.

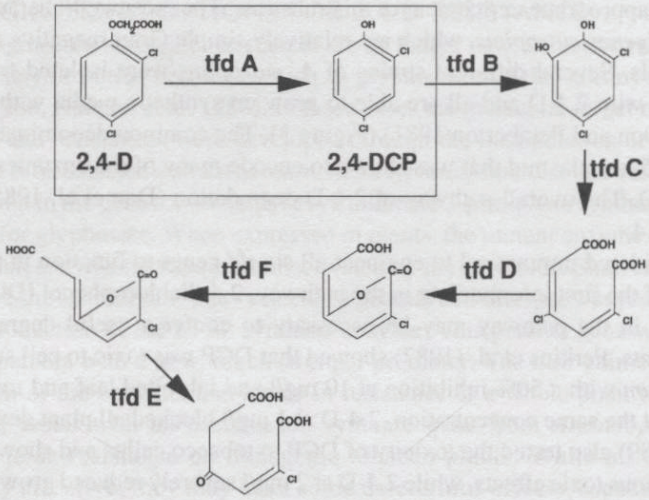

FIGURE 4 Biochemical pathway of 2,4-D degradation encoded by the degradative plasmid pJP4 of *Alcaligenes eutrophus*. 2,4-D degradative genes are designated *tfdA-F*, and the biochemical reaction catalyzed by their gene product is noted at each step. The 2,4-D dioxygenase reaction catalyzing the first breakdown step is boxed.

5. GENETIC ENGINEERING OF THE *ALCALIGENES TFDA* GENE FOR EXPRESSION IN PLANTS

Three research groups have engineered the *tfdA* gene for expression in plants (Streber and Willmitzer 1989, Lyon et al. 1989, Bayley et al. 1992). Sequencing of the *tfdA* gene revealed that it had an unusual translational start codon. This bacterial coding region began with a GTG codon rather than the usual ATG, a situation found in some other bacterial genes such as *tfdC* and *-D* (Perkins et al. 1990) and the *bar* gene from *Streptomyces hygrosopicus* (Thompson et al. 1987). The novel codon is not correctly recognized in plants (Lyon et al. 1989) and had to be modified to an ATG before reasonable expression levels could be attained. The modification of the translation start appeared to have no effect on 2,4-D dioxygenase enzyme activity in *Escherichia coli* (Lyon et al. 1989). Streber and Willmitzer and Lyon et al. also modified the region upstream of the ATG to more

closely resemble the consensus sequence compiled from a large number of plant genes (Joshi 1987). Bayley et al. (1992) used a *tfdA* gene derived from the *A. eutrophus* 2,4-D degradative plasmid pJP5 (Don and Pemberton 1981) rather than the pJP4 plasmid used by the earlier researchers. pJP5 has some restriction site polymorphisms relative to pJP4, but is very similar in size. No sequence data were presented for the pJP5 *tfdA* gene, but it is highly likely that it is nearly identical to the gene from pJP4, or at least had a common origin.

In order to express the 2,4-D dioxygenase gene in plants it was necessary to provide a strong plant promoter and transcription termination and polyadenylation signals. The latter were provided by a fragment of the nopaline synthase gene originally from the T-DNA of *Agrobacterium tumefaciens* (Herrera-Estrella et al. 1983) or a similar region from the T-DNA octopine synthase gene (Dhaese et al. 1983). All three groups used the promoter sequences from the 35S promoter of the cauliflower mosaic virus (CaMV), a strong and essentially constitutive promoter in many dicot plants (Odell et al. 1985). Two additional modified 35S promoters were used by Lyon et al. (1989) in an attempt to improve on the expression of the viral promoter. In the first, two additional copies of the so-called upstream enhancer region of the 35S promoter (the region between -90 and -340) were ligated onto the 35S promoter at -340 relative to the transcription start site. All three enhancer regions were in the same orientation. Kay et al. (1987) suggested that multiple copies of this enhancer increase the level of expression of the promoter more than arithmetically. In the second construction an additional 45 bp was added to the small leader region of the 35S promoter fragment (essentially only 2 bp). The addition consisted of the leader region of another viral gene, the coat protein gene of the alfalfa mosaic virus (AMV). This AMV leader has been suggested to improve the translational competitiveness of viral genes at least *in vitro* and perhaps also heterologous genes to which it is attached (Jobling and Gerhke 1987). Streber and Willmitzer also constructed a chimeric *tfdA* gene with the promoter from a light-inducible gene ST-LS1 (Stockhaus et al. 1987) that is expressed in all photosynthetically active tissues of a plant.

All chimeric *tfdA* genes were cloned either directly into binary plant transformation vectors such as pGA470, developed by An et al. (1985), or pBin19, developed by Bevan (1984), which was conjugally transferred into disarmed strains of *Agrobacterium tumefaciens*, or they were cloned into an intermediate vector that was transferred to *Agrobacterium* and recombined into a disarmed Ti plasmid. The binary plasmids are self-replicating and carry left and right T-DNA borders that direct integration into the plant genome, and a selectable kanamycin resistance gene that allows the recovery of transformed plant cells. The transfer functions are provided by modified Ti plasmids deleted for their own T-DNA. Lyon et al. used *Agrobacterium* strains LBA4404 (Hoekema et al. 1983) for transformation of tobacco and AGL1 (Lazo et al. 1991) for transformation of cotton. Bayley et al. used the octopine *Agrobacterium* strain GV3111 (containing a disarmed pTiB6S3) for both tobacco and cotton transformation. The system used by Streber and Willmitzer also used a kanamycin resistance gene as a selectable marker for tobacco transformation, but the chimeric *tfdA* gene, selectable marker, and T-DNA borders were present on the disarmed Ti plasmid GV3850Kan (Jones et al. 1985) rather than on a separate replicon in the *Agrobacterium* strain.

6. EXPRESSION OF THE 2,4-D DIOXYGENASE IN TRANSGENIC TOBACCO

Although none of the researchers were interested in 2,4-D-tolerant tobacco *per se*, tobacco provides a useful model system with which to test the function and specificity of introduced transgenes. Transgenic tobacco plants were produced by *Agrobacterium* infection of leaf pieces using relatively standard protocols with selection on toxic levels of kanamycin sulfate. Streber and Willmitzer (1989) screened 25 plants for production of nopaline (produced from an intact nopaline synthase gene present on the T-DNA) and for expression of the *tfdA* transcript in Northern blot

analyses. Of the 25 plants, 20 were producing detectable levels of the expected 1.3-kb chimeric *tfdA* transcript. Lyon et al. (1989) produced >30 transgenic tobacco from each of their three *tfdA* constructs and carried out preliminary screening with a radioactive enzyme assay using ^{14}C-labeled 2,4-D. While this assay worked well with bacterial extracts, it was only semiquantitative with plant extracts, but allowed the selection of the plants expressing the highest levels of the *tfdA* gene product. Since then the designation of the *tfdA* gene product has changed from a monooxygenase to a dioxygenase, new cofactor requirements have been discovered (Fukumori and Hausinger 1993), and it is likely that the original assay could be improved with the addition of ferrous ions and α-ketoglutarate. Bayley et al. (1992) also used a biochemical assay to measure expression of the *tfdA* gene product in transgenic tobacco, but they used whole cells (protoplasts) where endogenous cofactors would be present. They demonstrated an increase in $^{14}CO_2$ production from degraded ^{14}C-labeled 2,4-D in transgenic plants, ranging from 11 to 85 times higher than that from untransformed control tobacco tissues.

2,4-D has a drastic effect on plant growth and development, and when incorporated into tissue culture media can drastically perturb the effects of other hormones in the medium. When added to regeneration medium shoot development can be inhibited, and this provided a convenient assay procedure for ranking the 2,4-D tolerance of different transgenic plants. Streber and Willmitzer placed explanted leaf disks from their transgenic tobacco onto a medium containing 1 mg/l benzyl amino purine supplemented with varying amounts of 2,4-D from 0.1 to 4.0 mg/l. Nontransgenic plants would produce shoots on medium without 2,4-D, but shoot regeneration was inhibited by 0.2 mg/l 2,4-D. Depending on the individual transgenic plant, shoot regeneration was not inhibited by as much as 2 to 4 mg/l 2,4-D for plants transformed with a 35S-*tfdA* gene construct, indicating at least a 20-fold increase in tolerance to this herbicide. Plants transformed with the *tfdA* gene driven by the light-inducible promoter showed no more tolerance to 2,4-D than nontransformed tissues in this shoot regeneration assay, probably because the light-inducible promoter was not functional during the early stages of shoot morphogenesis, when growth is essentially nonphotosynthetic. In a similar assay Lyon et al. (1989) were able to produce plants with a tolerance to as high as 12.8 mg/l 2,4-D, representing a 32-fold increase in tolerance over a nontransformed control. The increased resistance in different transgenic plants varied from 10- to 30-fold over the control plants. In most cases the resistance in the leaf disk assay correlated well with the intensity of the DCP band in the radioactive enzyme assay. Genomic Southern analysis on some of the plants indicated that most contained single-copy, or at most two-copy, insertions of the 35S-AMV-*tfdA*-Nos chimeric gene described in this study. Bayley et al. (1992) used the shoot inhibition assay to select for the highest expressing tobacco plants and were able to recover seven primary transformants that could produce shoots on 6 mg/l 2,4-D and two others that could produce shoots on 4 mg/l 2,4-D.

In order to look at the segregation of the 2,4-D resistance gene in progeny Streber and Willmitzer tested the germination of 2,4-D-tolerant lines on medium containing 0 to 0.1 mg/l 2,4-D, but observed no significant difference in the sensitivity between transformed and nontransformed plants. Lyon et al. (1989), however, germinated F1 progeny seeds on a suitable germination medium containing from 0 to 3.2 mg/l 2,4-D and saw significant differences between controls and plants that showed high tolerance in the shoot inhibition assay. Seeds of control plants failed to germinate, or, if cotyledons emerged, failed to elongate on as little as 0.2 mg/l 2,4-D. Transgenic plants expressing 2,4-D dioxygenase activity, on the other hand, germinated and elongated on as much as 3.2 mg/l 2,4-D. In some progeny sets >90% of the seeds germinated and continued to grow, suggesting multiple gene insertions, while in others there seemed to be the expected three-to-one segregation of a single-copy insertion of a dominant character. At higher levels of 2,4-D there were some seeds obviously more resistant than the rest, and it seems likely that these were the homozygotes.

Selected transgenic tobacco lines or progeny from plants showing increased tolerance in the shoot inhibition assay were clonally or sexually propagated and tested in the glasshouse for increased tolerance to the herbicidal action of 2,4-D sprayed to simulate a field application. Streber

and Willmitzer (1989) sprayed clonally propagated plants after 2 weeks' growth in the soil with 2,4-D at doses equivalent to 1, 3, and 10 kg/ha (10 kg/ha was 4 ml of a 1% aqueous solution of 2,4-D sodium salt). Plant damage was assessed after 2 to 3 weeks. A dose of 1 kg/ha totally inhibited growth of nontransgenic control plants. A plant transformed with the 35S-*tfdA* construct was resistant to 10 kg/ha, while a plant transformed with the light-inducible promoter-driven *tfdA* gene was resistant to 1 kg/ha 2,4-D even though it showed no increased tolerance in the shoot inhibition assay. Lyon et al (1989) sprayed F1 progeny seed germinated in soil in small pots (four seedlings per pot) and grown in a containment glasshouse until they were 6 weeks old. Groups of 16 plants each were sprayed with a commercial preparation of 2,4-D isopropyl ester in 50% acetone at concentrations from 0 to 3000 ppm (Figure 5). Control plants sprayed with as little as 30 ppm began to show symptoms after only 3 d when they began to wilt and show abnormal twisting of the stems. Many of the transgenic plants only began to show these same symptoms at 3000 ppm. Some of the transgenic plants were affected more than others, presumably due to the segregation of the 2,4-D resistance gene among the F1 progeny. After about 3 weeks control plants sprayed with >100 ppm were dead or dying, while many of the clearly resistant transgenic plants had grown out of the effects of the herbicide even when sprayed at 3000 ppm. While it is difficult to estimate the equivalent field application rate corresponding to our experimental sprayings we estimate that resistance to 3000 ppm in our system corresponds to at least 3 to 6 kg/ha, which is at least 6- to 12-fold above normal field application rates for 2,4-D on cereals (around 0.5 kg/ha). Bayley et al. (1992) sprayed clones of their primary transformants with 10 ml of either 0.2, 0.5, and 1% aqueous solutions of 2,4-D with estimated dosages of 4, 10, and 20 kg/ha, although this may be on the high side. Many of their plants survived even the 20 kg/ha dose when assessed after 3 weeks.

A. eutrophus JMP134 is able to degrade other substituted phenoxyacetate compounds than 2,4-D, so Streber and Willmitzer (1989) tested their transgenic plants for cross-resistance to some other phenoxyacetate herbicides using the shoot inhibition tissue culture assay. They saw a tolerance similar to that seen with 2,4-D when 4-chlorophenoxyacetic acid was included in the medium and

FIGURE 5 2,4-D dioxygenase gene expressed in transgenic tobacco confers tolerance to spraying with 2,4-D. Plants were sprayed with 1000 ppm 2,4-D isopropyl ester in 50% acetone until dripping wet. Damage was scored after 3 weeks. Transgenic 2,4-D-tolerant tobacco line is on the left and control sensitive tobacco on the right.

only a slight increase in tolerance with MCPA, but no increased tolerance to 2,4,5-T or any of the phenoxypropionic acid herbicides [2-(4-chlorophenoxy)propionic acid. 2-(2,4- dichlorophenoxy)-propionic acid, or 2-(2,4,5-trichlorophenoxy)propionic acid]. Similar results were reported by Lyon et al. (1989) using a seed germination assay. These data are consistent with the known specificity of the *tfd* degradative plasmid of *A. eutrophus* for these differently substituted phenoxyacetates. The enzyme specificity also means that the 2,4-D dioxygenase will not degrade natural plant auxins, such as indole acetic acid (Figure 1), and hence is not toxic to the plants.

7. TRANSGENIC COTTON SHOWING TOLERANCE TO 2,4-D

While there are several crops that could benefit from being made tolerant to the herbicide 2,4-D, little interest has been shown in any but cotton. Perkins et al. (1987) expressed interest in using a *tfdA* gene to confer 2,4-D tolerance on the legume cowpea (*Vigna sinensis*), but although they cloned a duplicated *tfdA* gene (*tfdAII*) from the *Alcaligenes* plasmid pJP4 (Perkins and Lurquin, 1988), they have not continued the project (Lurquin, personal communication), mainly because of concerns over the safety of 2,4-DCP. With this in mind, they cloned and sequenced the genes encoding later steps of the 2,4-D breakdown pathway, *tfdB* (encoding 2,4-DCP hydroxylase) and the chlorocatechol oxidative operon *tfdCDEF* (Perkins et al. 1990) with the idea of using these genes in transgenic plants.

Cotton has been the only commercial crop that has been engineered for 2,4-D tolerance, although it has been difficult, as has the regeneration of cotton plants from tissue culture, let alone transformation. Regenerability is highly genotype dependent and it was not until recently that reliable regeneration via somatic embryogenesis could be achieved (Gawel et al. 1986, Trolinder and Goodin 1987), and then predominantly with "Coker" varieties of cotton. Transformation was reported for Coker cottons using *Agrobacterium*-mediated gene transfer and regeneration from either seedling cotyledon (Firoozabady et al. 1987) or hypocotyl (Umbeck et al. 1987) explants. Transformed callus tissue was selected on media containing kanamycin at relatively low levels (25 to 35 μg/ml) and somatic embryos induced from the callus. These embryos were germinated on appropriate media and grown to maturity in soil. The frequency of transformation even with Coker cotton is still very low but managable. Cousins et al. (1991) developed a tissue culture system and transformation procedures for two commercial Australian cotton cultivars, offering the possibility for direct transformation of elite lines.

Bayley et al. (1992) transformed Coker 312 cotton with the *tfdA* gene from pJP5 driven by the 35S promoter, and regenerated 300 transgenic plantlets from 30 independent cell lines. They prescreened them for increased tolerance to 2,4-D by selection for rooting on medium containing 0.05 mg/l 2,4-D or by treating meristems with 0.01 mg/l 2,4-D. These levels inhibited new root and shoot development on nontransformed control plants. Three transformed lines that survived these treatments were transferred to soil for further analysis, and vegetatively propagated clones also tested for increased tolerance to commercial formulations of 2,4-D. Many of the primary transformants were unaffected by a dosage (1.5 kg/ha) of three times the normal field application rate, whereas control plants were severely affected by a level 300 times less than the recommended dose. Selfed progeny of some lines were assayed for 2,4-D degradation using the protoplast whole cell assay for 2,4-D dioxygenase and were shown to have between 17- and 38-fold the activity present in nontransformed cotton protoplasts. Germination assays showed that these plants could form roots on medium containing 0.1 mg/l 2,4-D, a level that inhibited germination of nontransformed plants. This assay also was used to show three-to-one segregation of the resistance phenotype in one of the transformed lines. Seedlings from the same line when germinated in the absence of 2,4-D, were sprayed with 2,4-D at 1.5 kg/ha, and 83 of the 107 plants sprayed survived this treatment. Transgenic seeds are being distributed to public and private breeders in the U.S. for incorporation of the trait into other commercial germplasm (Trolinder, personal communication).

Lyon et al. (1993) transformed Coker 315 cotton with one of the *tfdA* gene constructs used to transform tobacco and regenerated 201 plantlets from 11 different tissue culture lines. Only four of these produced self-fertile plants when transferred to the glasshouse. Southern analysis and progeny testing indicated that only one of the four lines had a single T-DNA insert, while the others had two copies of the T-DNA. There was no direct correlation between copy number and the level of resistance shown by spraying plants with 2,4-D. 2,4-D dioxygenase expressing progeny from all four lines was undamaged by 300 ppm 2,4-D isopropyl ester in 50% acetone (Figure 6). The line containing a single copy of the chimeric gene was shown to be the most tolerant and was resistant to over 600 ppm 2,4-D. T2 and T3 plants shown to be either hemizygous or homozygous indicated that at discriminating doses the resistance phenotype is semidominant (Lyon et al. 1993). When sprayed with 600 ppm 2,4-D the hemizygous plants containing a single gene copy are slightly damaged, but eventually grow out of the damage and have a normal yield and appearance. Homozygous plants are completely unaffected by this level of 2,4-D, but show some minor damage at 900 ppm and more severe damage at 1200 ppm, although they survive this higher dose. The best transgenic plants were estimated to be about 100-fold more tolerant to 2,4-D than control nontransformed cotton and were tolerant to about twice the normal field application rate. CSIRO is currently backcrossing the 2,4-D tolerance developed by Lyon et al. (1993) into important commercial Australian cultivars to provide them with spray drift protection from 2,4-D.

None of the 2,4-D-tolerant plants have progressed to field trials. In Australia the research group at CSIRO is continuing to develop new gene constructs that may have better 2,4-D tolerance levels in cotton. Because the site of action of the herbicide is at the shoot meristem they are investigating

FIGURE 6 2,4-D-tolerant cotton cultivars engineered to express the *A. eutrophus* 2,4-D dioxygenase gene. Primary Coker transformants and controls were sprayed with 300 ppm 2,4-D isopropyl ester in 50% acetone. The plant on the left contains a single T-DNA insertion of the *tfdA* gene and was unaffected by 2,4-D at the concentration tested. The plant on the right is a nontransformed control cotton that was rapidly killed by the herbicide.

using gene promoters other than the 35S promoter, which has relatively poor expression in the meristem. Promoters from genes, such as histones and ubiquitin that are expressed at high levels in actively dividing cells, were linked to the *tfdA* gene and are currently being assessed in transgenic tobacco. Expression of the second gene in the 2,4-D degradation pathway, 2,4-D hydroxylase (*tfdB*) (Perkins et al. 1990), is also being considered to detoxify the DCP produced by the 2,4-D dioxygenase. Increased tolerance to 2,4-D will provide a greater protection margin and may allow 2,4-D to be used directly on cotton to control problem broadleaf weeds.

8. 2,4-D-TOLERANT COTTON PLANTS — AN ETHICAL DILEMMA?

The ability to produce 2,4-D-tolerant crops such as cotton raises the ethical dilemma of whether these plants should be used in agriculture. Opponents of herbicide-tolerant crops warn that herbicide-tolerant plants will promote indiscriminant usage of herbicides with concomitant human health and environmental impacts (Goldburg et al. 1990; Phelps 1990), but are these fears justified with 2,4-D?

As 2,4-D has been used in agriculture for almost 50 years it sometimes has been labeled as an "older chemical," and like several other older chemicals it has acquired a tainted image as a suspected environmental and human health hazard. The name 2,4-D has become associated in the public mind with less benign chlorinated hydrocarbons such as DDT (dichlorodiphenyl-trichloroethane). Unlike DDT, which accumulates in body fat, 2,4-D is rapidly excreted in an unchanged form from the human body. The biological half-life of 2,4-D in humans is approximately 18 to 20 h (reviewed by Ibrahim et al. 1991). Similarly, 2,4-D is unlikely to accumulate in aquatic organisms, as exemplified by the rapid renal elimination of 2,4-D administered to channel catfish (Plakas et al. 1992). This contrasts markedly with DDT, which is concentrated manyfold in the tissues of aquatic organisms, especially shellfish. 2,4-D is also rapidly broken down in the soil by bacteria and fungi, typically becoming undetectable about 5 to 6 weeks after application (Nobel and Hargreaves 1992). Soils with a history of 2,4-D treatment were shown to have an increased population of 2,4-D-degrading microorganisms (Holben et al. 1992). The unpopularity of 2,4-D in some circles stems in part from its inclusion along with 2,4,5-T in Agent Orange. However, the human health hazard posed by Agent Orange was caused by a contaminant, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, an extremely toxic compound which causes a severe form of acne and is suspected of causing birth defects and specific cancers. Modern 2,4-D formulations do not contain this dangerous contaminant nor do they contain significant quantities of any other known carcinogens (Ibrahim et al. 1991).

2,4-D has a low acute oral or dermal toxicity. The Western Australian Department of Agriculture lists 2,4-D as "slightly toxic" (poison schedule S5), placing it in the same category as glyphosate, sodium chlorate, and kerosene. The LD_{50} values for 2,4-D (reviewed in Ibrahim et al. 1991) range from 100 mg/kg in dogs (which are known to excrete organic acids more slowly than rodents or humans) to 1000 mg/kg in guinea pigs. Chronic exposure to 2,4-D in rats did not produce any observable detrimental effects at levels of up to 30 mg/kg/d. Studies reviewed by Mullison (1988) concluded that 2,4-D is not carcinogenic in mice at up to 45 mg/kg/d, nor is it teratogenic in rats even at a dosage that caused toxic symptoms in pregnant rats (75 mg/kg/d). There has been a suggestion, based on epidemiological evidence, that exposure to 2,4-D may be linked with the occurrence of the human cancer non-Hodgkin's lymphoma (Woods et al. 1987; Zahm et al. 1990), and this has been the subject of much debate. A panel of 13 scientists reviewing the literature were unable to reach a single conclusion as to whether 2,4-D is a human carcinogen (Ibrahim et al. 1991). Of the 13, five thought that it was a possible but unlikely human carcinogen, while one considered

that it was a possible carcinogen. Two panelists thought that it was unlikely that 2,4-D can cause cancer in humans. Following a report of a possible link between 2,4-D and canine malignant lymphoma (Hayes et al. 1991), a scientific review panel examined the available evidence and concluded that the use of 2,4-D by dog owners did not show an association with the occurrence of canine malignant lymphoma (Carlo et al. 1992). In other words, there is no conclusive evidence to implicate 2,4-D as a carcinogen.

The *Agrochemicals Handbook* of the Royal Society of Chemistry (Hartley and Kidd 1983) lists 2,4-D as nontoxic to fish (average lethal concentration of sodium salt, 1160 mg/l/24 h) and nontoxic to bees. 2,4-D is only toxic to waterfowl at very high concentrations (LD_{50} = 2000 mg/kg; Weiss 1986), which would never be approached by the use of it in agriculture.

2,4-D has many of the desirable qualities sought in a herbicide, most notably its low toxicity to animal life and high biodegradability. Furthermore, it is very inexpensive and effective. Its use over many years has been relatively untroubled by problems of resistance development in weeds. There are only six sites in the world where 2,4-D-resistant weeds are thought to have evolved as a result of spraying, compared to over 1000 sites for herbicides such as the triazines and acetolactate synthase inhibitors (Holt et al. 1993). 2,4-D does, however, have a drawback in that it does not adsorb readily to some soils and can leach into groundwater. Precautions should be taken to ensure that pollution of ground water does not occur.

Like any agricultural chemical, 2,4-D should not be used carelessly, but the large body of toxicological data gathered over the past 40 or so years suggests that it can be used according to label directions without endangering humans or the environment.

9. CONCLUSIONS

The herbicide detoxification systems developed for the protection of cotton against 2,4-D have proven to be extremely effective in at least two plant species, and we expect that it also will be useful in other crops that are sensitive to 2,4-D. The plants containing the engineered resistance gene appear to grow and flower normally, producing just as many viable seeds as nontransformed plants, and there appears to be no yield penalty in the absence of any applied herbicide, at least in glasshouse trials. When sprayed at relatively high levels of 2,4-D there is a slight retardation of growth, but the plants recover quickly. Because most commonly used herbicides are broken down by soil microorganisms it would seem feasible to apply the same principles to those herbicides and to quickly develop a whole range of herbicide resistance genes that could, once transformation procedures become routine, revolutionize farming practices in high-input crops such as cotton.

Herbicide-tolerant crop projects, including the 2,4-D-resistant cotton projects described here, have been criticized by environmental groups, such as the Australian Conservation Foundation (Phelps 1990), on the grounds that they could lead to an increase in the use of synthetic chemicals in agriculture. This fear should be balanced with the environmental benefits of being able to extend the spectrum of use of relatively benign herbicides such as 2,4-D and thereby reduce our reliance on more environmentally damaging herbicides. Additionally, control of weeds within the crop with 2,4-D could potentially lead to a no-till cropping system which would have great benefits in reducing soil erosion, providing a mulch, and returning organic nitrogen to the soil.

Although widely held public concern about the use of 2,4-D-resistant cotton are not supported by scientific data, it is nevertheless essential that careful studies in the field are carried out in order to evaluate any possible environmental implications. This will enable cropping systems to be designed so that the new technology can be applied responsibly in a way that benefits the farmer, the consumer, and the environment.

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