

Expression of a bacterial gene in transgenic tobacco plants confers resistance to the herbicide 2,4-dichlorophenoxyacetic acid

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Received 1 June 1989; accepted 15 June 1989

Key words: 2,4-dichlorophenoxyacetic acid (2,4-D), detoxification, herbicide resistance, transgenic plants

Abstract

Plants resistant to the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) were produced through the genetic engineering of a novel detoxification pathway into the cells of a species normally sensitive to 2,4-D. We cloned the gene for 2,4-D monooxygenase, the first enzyme in the plasmid-encoded 2,4-D degradative pathway of the bacterium *Alcaligenes eutrophus*, into a cauliflower mosaic virus 35S promoter expression vector and introduced it into tobacco plants by *Agrobacterium*-mediated transformation. Transgenic tobacco plants expressing the highest levels of the monooxygenase enzyme exhibited increased tolerance to 2,4-D in leaf disc and seed germination assays, and young plants survived spraying with levels of herbicide up to eight times the usual field application rate. The introduction of the gene for 2,4-D monooxygenase into broad-leaved crop plants, such as cotton, should eventually allow 2,4-D to be used as an inexpensive post-emergence herbicide on economically important dicot crops.

Introduction

The introduction of novel traits such as herbicide resistance into agronomically important crop plants is one promising aspect of the application of genetic engineering to plant breeding. Herbicide-tolerant transgenic plants have been produced in a variety of ways, including over-expressing a sensitive target gene product or supplementing the sensitive plant cells with mutant, herbicide-resistant copies of the target gene. Detoxification strategies, in which bacterial genes for herbicide detoxification or degradation have been used (e.g. degradative genes for phosphinothricin [3] and bromoxynil [19] detoxification), are an alternate and more straightforward means of creating her-

bicide-resistant plants since it is unnecessary to first identify the biochemical target of the herbicide. This is particularly important where the effect of the herbicide may be pleiotrophic, as in the case of 2,4-dichlorophenoxyacetic acid (2,4-D), a compound which mimics the action of the plant hormone auxin.

Soil organisms that degrade 2,4-D were identified more than thirty years ago [20] and multi-enzyme pathways for 2,4-D degradation have subsequently been demonstrated in several bacterial genera [4, 8, 22]. In the most intensively studied organism, *Alcaligenes eutrophus*, the 2,4-D degradative pathway comprises six specialized enzymes, encoded by a 75 kb plasmid designated pJP4, which convert 2,4-D to chloromaleylacetic

acid, a compound subsequently metabolized to succinic acid [5]. The genes for these six enzymes (*tfdA-F*) have been mapped by transposon insertion and the sequences of three of the genes (*tfdA*, *tfdC* and *tfdD*) have been determined [5, 11, 21].

The first gene in the 2,4-D degradation pathway (*tfdA*) encodes a 2,4-D monooxygenase which converts 2,4-D into 2,4-dichlorophenol by cleavage of the aliphatic side chain [21]. Preliminary evidence on the biological effects of 2,4-dichlorophenol suggests that this degradation product is less toxic to plants than the herbicide [18]. Accordingly, we cloned the gene for the 2,4-D monooxygenase (*tfdA*), transferred it into tobacco plants and demonstrated that transgenic plants expressing this enzyme exhibit tolerance to the herbicide 2,4-D.

Materials and methods

Manipulation of the 2,4-D monooxygenase gene (*tfdA*)

Alcaligenes eutrophus strain JMP134 carrying the 2,4-D degradative plasmid pJP4 was generously provided by R.H. Don (University of Queensland, Australia). Following conjugative transfer to *Escherichia coli* MC1061, plasmid DNA was isolated, cleaved with *Hind* III and cloned into the *Hind* III site of pUC119 by standard techniques [16]. A transformant bearing the 21 kb *Hind* III B fragment of pJP4 cloned in pUC119 then served as the source of DNA for further manipulation of the *tfdA* gene. The *Hind* III fragment was reduced by successive digestion with *Bam* HI, *Sal* I, *Xba* I and *Nco* I to give a 980 bp fragment bounded by *Bam* HI linkers (GGGATCCC, Boehringer Mannheim) at the filled-in *Xba* I and *Nco* I sites (Fig. 1A). Analysis of the *tfdA* sequence [21] had revealed the presence of an out-of-frame ATG codon 95 bp upstream from the GTG translational start codon, and this sequence was removed by BAL-31 digestion [16] of 20 bp from the 5' end of the subclone. Finally, oligomutagenesis was

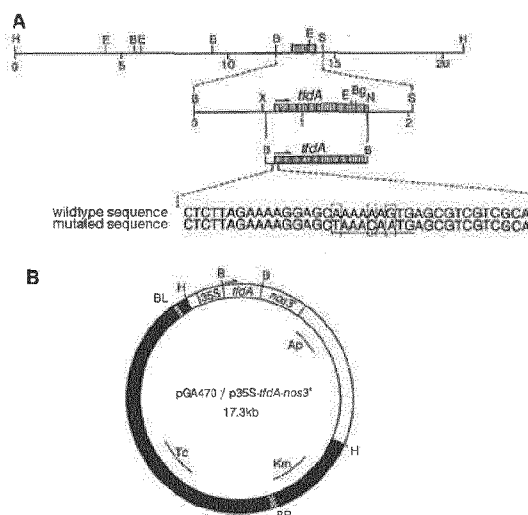


Fig. 1. Cloning of the gene for 2,4-D monooxygenase (*tfdA*) from *Alcaligenes eutrophus* strain JMP134. A. The 960 bp *Bam* HI-*Bam* HI fragment was produced from the 21 kb *Hind* III B fragment of plasmid pJP4 by successive subcloning and BAL-31 digestion as described in Materials and methods. This clone was subjected to oligomutagenesis in the vicinity of the bacterial GTG translational start codon (base changes are boxed) to produce a plant consensus sequence and ATG translational start codon (underlined). The scale beneath each map is in kilobase pairs. Restriction endonuclease sites are: B, *Bam* HI; Bg, *Bgl* II; E, *Eco* RI; H, *Hind* III; N, *Nco* I; S, *Sal* I; X, *Xba* I. The arrow indicates the direction of translation of *tfdA*. B. Schematic representation of the *tfdA* gene cloned between the cauliflower mosaic virus 35S promoter and the nopaline synthase (*nos*) 3' polyadenylation signal of pJ35SN and inserted into the binary vector plasmid pGA470. Km designates the neomycin phosphotransferase gene which mediates kanamycin resistance in transformed plants; Ap and Tc designate the bacterial genes for ampicillin and tetracycline resistance, respectively. BL and BR designate the left and right borders of the T-DNA, respectively.

conducted using an adaptation of an oligonucleotide deletion method [6] to convert the GTG start codon to an ATG and to produce a sequence which matches a published plant consensus for the region adjacent to the translation start [14]. The 37 base oligonucleotide CTCTTAGAA AAGGAGCTAAACAATGAGCGTCGTC GCA was synthesized using an Applied Biosystems 380A DNA synthesizer and correct mutagenesis of the start codon region confirmed by dideoxy sequencing.

Construction of gene fusions

The *tf₁* gene was excised as a 960 bp *Bam* HI fragment and cloned into the unique *Bam* HI sites of three plant expression vectors based on the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase (*nos*) 3' polyadenylation signal. Plasmid pJ35SN was constructed by replacing the *Hind* III-*Bam* HI *nos* promoter-chloramphenicol acetyltransferase coding sequences of pNCAT4 (obtained from L. Herrera-Estrella and M. Van Montagu, Rijksuniversiteit, Gent, Belgium), with a *Hind* III-*Bam* HI fragment containing the 35S promoter of p35SCN [23], and subcloning a *Sca* I-*Hind* III fragment derived from this construct (comprising 35S promoter and *nos* 3' separated by *Bam* HI, *Sma* I and *Eco* RI sites) into *Sca* I-*Hind* III cleaved pUC19. Plasmid p3x35SN was constructed by cloning two additional copies of the 35S promoter upstream enhancer region [15], bounded by *Sal* I and *Eco* RV sites, 5' to the 35S promoter of pJ35SN. All three enhancer regions were in the same orientation. Plasmid p35SAMVN was constructed by cloning a synthesized 44 base oligonucleotide equivalent to the alfalfa mosaic virus (AMV) leader [10] and bounded by *Bgl* II and *Bam* HI sites into the *Bam* HI site of pJ35SN. The unmodified GTG containing *tf₁* coding sequence was also cloned into pJ35SN in both orientations.

Construction of binary vectors and plant transformation

As illustrated in Fig. 1B, the 35S-*tf₁*-*nos* constructs were cleaved with *Hind* III and cloned into the *Hind* III site of the binary vector plasmid pGA470 [1]. Triparental mating with selection as described [7] was employed to transfer the binary vector constructs from *E. coli* to the avirulent *Agrobacterium tumefaciens* strain LBA 4404 Rif^r [12]. Constructions in *Agrobacterium* were verified by restriction and DNA blotting analysis. Leaves of *Nicotiana tabacum* cv. Wisconsin 38 were transformed by these *Agrobacterium* strains

and shoots were regenerated on kanamycin medium essentially as described [7]. Transgenic kanamycin-resistant plants transformed by these promoter constructs were designated TFD 1, 5 or 8 (corresponding to *tf₁* in the coding orientation in pJ35SN, p3x35SN or p35SAMVN, respectively), or TFD 3 (corresponding to *tf₁* in the inverse orientation in pJ35SN). Transgenic plants were maintained under antibiotic selection in aseptic containers and young leaves were periodically harvested for analysis. Selected transgenic plants were transferred to soil in a biosafety containment glasshouse and propagated for seed.

2,4-D monooxygenase assay

Bacterial enzyme extracts were prepared by sonication of cultures suspended in 0.02 M potassium phosphate buffer (pH 7.3), while plant enzyme extracts were prepared by grinding tissue with sand in the presence of 0.02 M potassium phosphate buffer, 2% insoluble polyvinylpyrrolidone and 10 mM dithiothreitol. Extracts were equilibrated for protein content and assayed for 2,4-D monooxygenase activity in the presence of 0.01–0.03 μ Ci 2,4-dichloro[ring-¹⁴C]phenoxyacetic acid (Amersham; purified by ethyl acetate extraction), 1 mM cysteine and 20 mM NADH. After being flushed with O₂, the assay mixes were incubated with shaking at 30 °C for 1–3 h. The reaction products were extracted into chloroform and separated by thin-layer chromatography (TLC) using benzene-methanol (19 : 1, v/v) as solvents [9]. Phenolic compounds were detected on the chromatogram by staining with diazotized *p*-nitroaniline followed by NaHCO₃ [2] or by autoradiography with Fuji X-ray film for 1–14 days.

2,4-D toxicity tests

Tobacco leaf discs of approximately 1 cm in diameter were punched aseptically from fresh leaves and plated on shoot regeneration media [17] containing 0.2, 0.8, 3.2 or 12.8 mg/l 2,4-D

and incubated for 60 days before shoot and callus tissue was weighed for comparison. Leaf discs from wild-type plants were routinely plated as internal controls on test plates. F_1 seeds generated by self-pollination from wild-type and transgenic plants were surface-sterilized and plated on modified Murashige and Skoog media (no vitamins, 10 g/l sucrose) containing 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 mg/l 2,4-D or 200 mg/l kanamycin and incubated for 14 days before relative germination rates were determined. At 4–8 weeks after germination, seedlings were sprayed until dripping wet with a range of 2,4-D (isopropyl ester in 50% acetone) concentrations (0, 10, 30, 100, 300, 1000 and 3000 mg/l) and were observed for detrimental effects over a period of three weeks.

Results

Cloning and expression of tfdA in bacteria and plants

The 2,4-D monooxygenase gene (*tfdA*) of *A. eutrophus* plasmid pJP4 possesses an 861-nucleotide open reading frame [21]. We cloned in *E. coli* a 960 bp segment comprising the complete *tfdA* gene bounded by 5' and 3' non-coding sequences of approximately 90 bp and 10 bp, respectively (Fig. 1A). Oligomutagenesis of three bases in the 5' region of the gene converted the GTG translational start codon of the native gene to an ATG and produced a plant consensus sequence [TAAACA] immediately upstream of this codon (Fig. 1A).

Both unmodified (GTG) and modified (ATG) *tfdA* genes were fused to the β -galactosidase promoter of pUC plasmids and expression of 2,4-D monooxygenase in *E. coli* was induced by culture in the presence of isopropylthiogalactoside (IPTG). In order to confirm that the cloned and mutated *tfdA* fragment did encode a fully active 2,4-D monooxygenase, bacterial cell extracts were assayed for their ability to convert ^{14} C-labelled 2,4-D into 2,4-dichlorophenol (Materials and methods). An abundant radiolabelled product was detected in extracts from bacteria harbouring either the unmodified or modified *tfdA*

gene (Fig. 2, lanes 2 and 3), but was not observed in the enzyme-free control (Fig. 2, lane 5), nor in extracts of *E. coli* containing pUC118 or the modified *tfdA* gene cloned in the inverse orientation (Fig. 2, lanes 1 and 4). This product exhibited a TLC R_F value of approximately 0.46 for the benzene-methanol solvent system and co-migrated with authentic 2,4-dichlorophenol (BDH). Sufficient quantities of the enzymic product were present to demonstrate the characteristic purple colour of 2,4-dichlorophenol by direct staining with diazotized p-nitroaniline.

Assays for 2,4-D monooxygenase activity were conducted on up to thirty different plants produced by transformation with each of the constructs and on the untransformed wild-type tobacco W-38. Unlike the *E. coli* extracts, the extracts from control tobacco plants were able to slowly metabolize 2,4-dichlorophenol by an, as yet, unknown mechanism. This assay was therefore not an accurate measure of the level of 2,4-D monooxygenase present in plant extracts, but did give a qualitative indication of the level of expression. Transgenic plants showing the highest level of 2,4-dichlorophenol were analysed further.

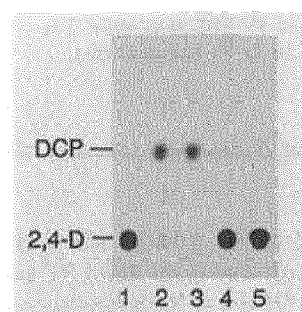


Fig. 2. Chromatogram of 2,4-D monooxygenase assay. Bacterial extracts were assayed for 2,4-D monooxygenase activity and the chromatogram was exposed to X-ray film as described in Materials and methods. Bands due to 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4-dichlorophenol (DCP) are marked. Lanes are: 1, *E. coli* (pUC118); 2, *E. coli* (unmodified *tfdA* cloned in pUC118); 3, *E. coli* (modified *tfdA* cloned in pUC119); 4, *E. coli* (modified *tfdA* cloned in incorrect orientation in pUC119); 5, enzyme-free control.

Leaf disc assay for resistance to 2,4-D

The increase in tolerance to 2,4-D *in vivo* was determined by assaying the ability of leaf discs from transgenic plants to regenerate shoots in the presence of the herbicide. Wild-type Wisconsin 38 tobacco leaf discs generated shoots on levels of 2,4-D up to 0.2 mg/l but were inhibited by more than 0.4 mg/l of the herbicide. At these higher levels of 2,4-D, large quantities of undifferentiated callus were produced, presumably as a response to the presence of the auxin analogue. In similar tests conducted with 2,4-dichlorophenol, W-38 tobacco-generated shoots at levels as high as 45–50 mg/l (above which tissue was bleached and killed), implying that 2,4-dichlorophenol is 100-fold less toxic to tobacco than the herbicide 2,4-D when applied externally.

A 2,4-D concentration of 0.8 mg/l, too toxic for the formation of shoots by wild-type tobacco, was used to test for transgenic plants tolerant to high levels of 2,4-D. This level of herbicide prevented the growth of shoots from explants of the non-transformed wild-type, inverse orientation of the ATG construction (TFD 3-1 plants), both orientations of the GTG constructions, together with at least one of the tested transgenic plants expressing low levels of monooxygenase (TFD 8-24). This level of 2,4-D did not adversely affect the formation of shoots by a number of transgenic plants, from all three different promoter constructs containing the ATG modified *tfdA* coding region, that expressed high levels of monooxygenase activity in the *in vitro* assay. Those plants containing the AMV leader region construction appeared marginally better, e.g. TFD 8-1, 8-2, 8-6 and 8-14 (Fig. 3), and these were analysed in most detail. Some of these transgenic plants generated shoots on levels of 2,4-D as high as 12.8 mg/l, representing a 32-fold increase in tolerance to the herbicide compared with the inverse orientation control TFD 3 and wild-type plants. Attempts to select directly for 2,4-D-resistant plants in transformation experiments were, however, unsuccessful.

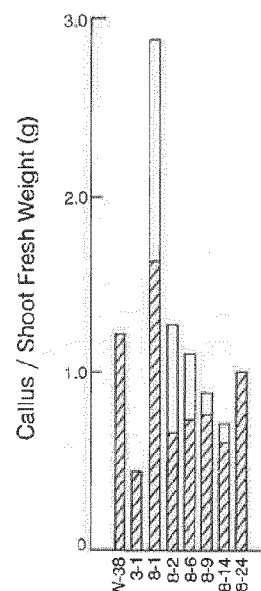


Fig. 3. Tobacco callus and shoot formation on media containing 2,4-D. Tobacco leaf discs were plated on shoot-inducing media containing 0.8 mg/l 2,4-D. Wild-type and transgenic callus (hatched) and shoot (open) formation is expressed as average fresh weight (g).

Seed germination assay for resistance to 2,4-D

Table 1 presents the germination data for seed derived by self-pollination of wild-type and transgenic plants. Germination of wild-type and control TFD 3 seed was affected by concentrations

Table 1. Germination of seed derived from wild-type and transgenic plants on 2,4-D and kanamycin expressed as a percentage of viable seed (50 seeds/treatment).

Plant	2,4-D (mg/l)						Kanamycin 200 mg/l
	0.1	0.2	0.4	0.8	1.6	3.2	
W-38	69	0	0	0	0	0	
TFD 3-1	57	0	0	0	0	0	60
TFD 1-1	85	54	62	52	4	0	80
TFD 5-2	86	41	0	0	0	0	96
TFD 5-10	90	78	68	40	0	0	68
TFD 5-27	89	73	64	73	9	0	80
TFD 8-1	92	67	63	69	33	0	94
TFD 8-6	92	92	81	65	22	10	92
TFD 8-14	80	74	66	50	4	0	60
TFD 8-24	52	10	0	0	0	0	96

of 2,4-D as low as 0.1 mg/l and was completely inhibited at 0.2 mg/l. In contrast, some transgenic seed was able to germinate on levels of 2,4-D up to 3.2 mg/l, an increase in tolerance to the herbicide of approximately 30-fold.

The seed germination assay was further employed to investigate cross resistance to other phenoxyacetic acid herbicides, including 2-methyl-4-chlorophenoxyacetic acid (MCPA) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). Seed from TFD 8-6, the most resistant in the plate assays, showed more resistance than wild-type seed to MCPA in the range 0.025–0.1 mg/l, and 20% of the transgenic seed was able to germinate at 0.2 mg/l, a level which prevented germination of the wild type. Transgenic seed was no more tolerant to 2,4,5-T than the wild-type seed.

2,4-D resistance in young plants

Progeny germinated from wild type, TFD 8-1 and TFD 8-6 seed were transferred to soil and grown for 4–8 weeks before being sprayed with concentrations of herbicide ranging from 0–3000 mg/l. Wild-type plants were affected by levels as low as 10 mg/l and were killed by levels of 300 mg/l or more (Fig. 4A). Transgenic plants were much more tolerant to the herbicide, being unaffected by 300 mg/l 2,4-D (Fig. 4A), only slightly affected by 1000 mg/l (data not shown), and even surviving 3000 mg/l 2,4-D (Fig. 4B).

An estimate of the field application rate corresponding to our experimental spraying regime suggests that 1000 mg/l is approximately equivalent to 1–2 kg of 2,4-D per hectare. For weed control, fields are normally sprayed with 0.25–0.5 kg/ha, hence, the transgenic tobacco F_1 progeny can survive spraying with 2,4-D concentrations 4–8 times the normal field application of the herbicide.

Discussion

The first gene in the 2,4-D degradative pathway of the bacterium *Alcaligenes eutrophus* (*tfdA*) was cloned into three different CaMV 35S promoter-

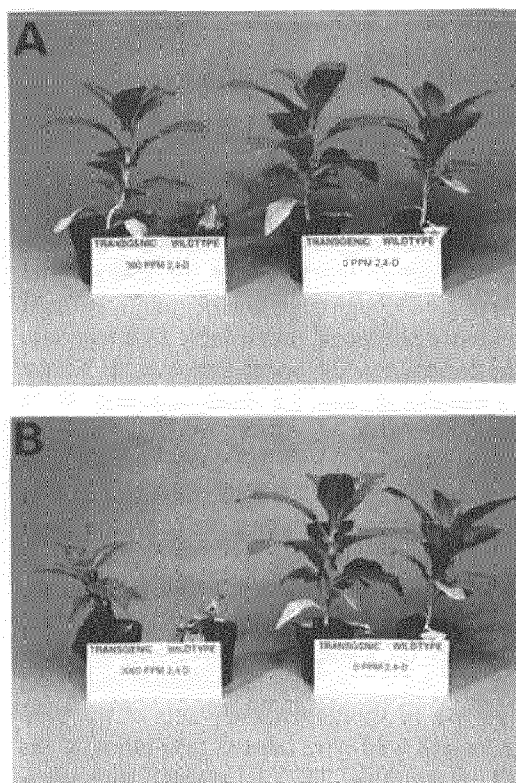


Fig. 4. Spraying of young tobacco plants with 2,4-D. Wild-type (W-38) and transgenic (TFD 8-6) plants were sprayed with (A) 300 mg/l 2,4-D, or (B) 3000 mg/l 2,4-D.

nopaline synthase 3' expression vectors, and introduced into tobacco plants by *Agrobacterium*-mediated transformation. The bacterial gene had been modified by oligomutagenesis to produce a plant consensus translation start sequence upstream of the translation initiation codon, changed from a GTG to ATG. Many of the transgenic plants demonstrated increased tolerance to 2,4-D as determined by the generation of shoots from leaf discs in the presence of high levels of the herbicide, while plants transformed with an unmodified *tfdA* gene did not exhibit improved tolerance to 2,4-D, indicating that this gene is not expressed efficiently in plants. Resistant plants were produced from all three promoter constructs. One of the most resistant contained the AMV leader region, purported to increase trans-

lational efficiencies of mRNAs containing this sequence [13]. More intensive studies will be required to fully evaluate the effectiveness of the different promoter constructs in transgenic plants.

A TLC assay for 2,4-D monooxygenase activity in bacterial extracts was not suitable for the accurate determination of enzyme activity in plant extracts, but did enable the initial selection of potential 2,4-D-resistant transgenic plants. A strong correlation was observed between this assay and the three *in vivo* methods (leaf disc, seed germination and spray toxicity) of assessing resistance to 2,4-D. An alternative assay, using antibodies to 2,4-D monooxygenase, is being developed for the early detection of enzyme in transformed tissue from cotton and other plants.

The seed germination assays suggest that high levels of resistance to the herbicide are dependent on both the possession of multiple copies of the *tfdA* gene and the site of integration of the T-DNA in the genome. Seed segregation ratios in the presence of discriminating doses of 2,4-D give some indication of the number of gene insertions in each transgenic plant and support Southern blot analyses of genomic DNA from the parental plants. These results indicate that more than one copy of the *tfdA* gene is present in the most resistant plants such as TFD8-6.

Transgenic seed was found to be slightly resistant to the herbicide MCPA, a result consistent with the observation that *A. eutrophus* can slowly metabolize this compound, which is structurally similar to 2,4-D. The fact that there was no cross-resistance to the closely related herbicide 2,4,5-T implies a high degree of substrate specificity for the 2,4-D monooxygenase. We are currently testing a wide variety of phenoxyacetate herbicides in order to establish the full herbicide resistance spectrum mediated by the 2,4-D monooxygenase and to gain insight into the protein-substrate interactions involved.

Although the level of resistance to 2,4-D in tobacco is adequate, with transgenic progeny able to survive spraying with 2,4-D concentrations some 4–8-fold higher than the usual field application rate, we expect that further improvement in

gene expression will be required to obtain adequate protection in other species which are often more sensitive to 2,4-D than tobacco. Despite using a strong promoter linked to the 2,4-D monooxygenase gene, we were unable to detect *tfdA* transcripts by Northern blot hybridization. It may be possible to increase expression by improving transcript stability, perhaps by shortening the non-translated leader sequence of the *tfdA* coding region fragment (currently 90 bp) or substituting new 3' ends in the expression vectors. These are strategies that have been effective with other gene constructs. The level of resistance in the current transgenic plants will be improved by selecting progeny carrying multiple copies of the *tfdA* gene and sequentially increasing the gene dosage by judicious crossing. This will be important to establish the upper limit of resistance possible with this enzymic detoxification system.

2,4-D is highly active against many species of broad-leaved weeds and is widely employed as a weed control agent on cereal crops. Broad-leaved crop plants, such as cotton, are sensitive to this herbicide, and accidental drift damage from the aerial spraying of neighbouring grain fields can devastate a crop. We are interested in engineering herbicide-resistant cotton that will firstly be resistant to 2,4-D drift damage, and secondly, will enable the post-emergence spraying of cotton with 2,4-D for the control of broad-leaved weeds. The result with the tobacco system suggests that the introduction and expression of the *tfdA* gene in cotton and other broad-leaved crops should provide the necessary level of herbicide tolerance to achieve these objectives.

Acknowledgements

This work was funded in part by Cotton Seed Distributors, Ltd. and the Australian Cotton Research Council. We thank Yvonne Cousins, Alan Downie and Hal Hatch for helpful suggestions, T.J. Higgins, Phil Larkin and Don Spencer for critical reading of the manuscript, and Jenny Thistleton and Les McCaffery for expert technical assistance.

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