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Engineering 2,4-D resistance into cotton

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Summary. To reduce damage by drift-levels of the herbicide 2,4-dichlorophenoxyacetic acid, we have engineered the 2,4-D resistance trait into cotton (*Gossypium hirsutum* L.). The 2,4-D monooxygenase gene *tfd*A from *Alcaligenes eutrophus* plasmid pJP5 was isolated, modified and expressed in transgenic tobacco and cotton plants. Analyses of the transgenic progeny showed stable transmission of the chimeric *tfd*A gene and production of active 2,4-D monooxygenase. Cotton plants obtained were tolerant to 3 times the field level of 2,4-D used for wheat, corn, sorghum and pasture crops.

Key words: Genetic modification – Gene transfer – *tfd*A – *Gossypium hirsutum* – Herbicide

Introduction

The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) and several related phenoxy compounds have been used extensively for more than 40 years to control broadleaf weeds. Since monocotyledonous plants are naturally tolerant to 2,4-D, it is applied to control weeds which grow among cereals, sugarcane, turf grass, and to manage forest undergrowth. Because 2,4-D is highly volatile and tends to drift in the prevailing winds, nearby crops that are susceptible are often damaged. One of the crops that is most susceptible to damage by this herbicide is cotton, and annual loss to 2,4-D damage in cotton is extensive. One approach to reducing these annual losses is to incorporate resistance to 2,4-D into cotton germplasm. Numerous strains of soil bacteria have been described which contain plasmid-borne genes that confer the ability to catabolize 2,4-D (Amy et al. 1985; Don and Pemberton 1981). The gene for the first step of 2,4-D degradation has been isolated from pJP4, a plasmid found in *A. eutrophus* (Streber et al. 1987; Lyon et al. 1989). This gene, *tfd*A, was shown to encode 2,4-D monooxygenase, which degrades 2,4-D into 2,4-dichlorophenol and glyoxylate. The transfer of modified versions of this gene into tobacco conferred resistance to the herbicide (Lyon et al. 1989; Streber and Willmitzer 1989).

In this communication, we report the engineering of 2,4-D resistance into cotton. A homologue *tfd*A gene was isolated from pJP5, another *A. eutrophus* 2,4-D degradative plasmid, and initially examined in tobacco. We show that both tobacco and cotton plants obtained by gene transfer exhibited 2,4-D degradative activity and were tolerant to high levels of the herbicide. Cultivation of 2,4-D tolerant varieties of cotton would reduce annual losses caused by drift-levels of the herbicide.

Materials and methods

Recombinant DNA

Standard techniques of recombinant DNA were used throughout (Maniatis et al. 1982). *A. eutrophus* DSM 4059 and *E. coli* DSM 4063 harboring plasmid pJP5 were obtained from the German collection of microorganisms (DSM, Mascheroder Weg 1 b, W-3300 Braunschweig, FRG). DNA oligonucleotides corresponding to the 5′- and 3′-coding regions, 5′-AGCGTCGTCGCAAATCCC-3′ and 5′-ATCGTCCAGGGTGGTCGC-3′, respectively, were used as synthetic probes to detect the *tdf*A gene by Southern hybridization. A partial genomic clone bank was constructed from DSM 4063 by inserting approximately 2 kb gel-purified XbaI and SalI doubly-digested DNA fragments into pUC19. Colony hybridization was used to detect *tfd*A-containing plasmids. The same primers were used for limited DNA sequence analysis of the 5′ and 3′ ends of the coding region to verify *tfd*A identity.

A modified *tfd*A gene was constructed for expression in plant cells. The approximately 860-bp 5′-FokI to NcoI-3′ frag-

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ment, obtained from partial digestion with FokI and containing the *tfd*A coding region except for the first 11 bp, was fused to a synthetic adapter

5′-CTAGACACAAAGTGGTTAACAAGGAAGGAAAAATGAGCGTCGT-3′
3′-TGTGTTTCACCAATTGTTCCTTCCTTTTTACTCGCAGCAGCGT-5′

and a SalI linker (5′-GGTCGACC-3′) at the 5′ and 3′ ends, respectively (Fig. 1). The 5′ end adapter regenerated the original *tfd*A coding region except for an ATG in place of GTG as the putative start codon. Preceding the coding region is a leader sequence with a prokaryotic ribosome binding site. This modified XbaI to SalI *tfd*A fragment was then inserted between the XbaI and SalI sites of pUC19, allowing excision of the modified *tfd*A gene as a BamHI/SphI fragment, which was then placed within the corresponding sites in pDO432 (Ow et al. 1986). This placed the modified *tfd*A gene between a 1585-bp 35S (CaMV 35S promoter) fragment and a 754-bp *nos*3′ (nopaline synthase gene polyA) fragment to yield pRO17 (Fig. 1). To transfer the *tfd*A gene into an *Agrobacterium* gene transfer vector, pRO17 was linearized by HindIII and ligated to HindIII-cleaved pBIN19 (Bevan 1984) to form a co-integrate plasmid, with transcription of the *tfd*A gene towards the left T-DNA border (Fig. 1). The cointegrate pBIN19::pRO17 was transferred into the "disarmed" *A. tumefaciens* strain GV3111 harboring the helper Ti plasmid pTiB6S3S3.

Gene transfer into tobacco and cotton

Agrobacterium-mediated gene transfer into *Nicotiana tabacum* (cv 'Wi38') has been described (Horsch et al. 1985). Kanamycin (100 μg/ml) was used to select transformed cells, and cefotaxime (500 μg/ml) was used to remove *Agrobacterium*. The same *Agrobacterium* vector was used for gene transfer into cotton (cv 'Coker 312'). Transformation of cotton was similar to that reported previously (Umbeck et al. 1987) with the following modifications. Hypocotyl sections were precultured for 3 days on MS salts (Gibco) containing B5 vitamins, 30 g/l glucose, 0.1 mg/ml 2,4-D and 0.5 mg/l kinetin. Kanamycin and cefotaxime were used at 50 μg/ml and 500 μg/ml, respectively. Plant regeneration was performed as described (Trolinder and Goodin 1987) except that embryos were partially dehydrated over a 2-week period by placing them on a 2% agar medium containing 15 ml of S medium (Stewart and Hsu 1977) and 30 g/l glucose. S medium containing 0.5% agar, 1.5 g/l Gelrite and 0.5% sucrose was used for embryo germination.

Assay for 2,4-D degradative activity

Bacterial cultures were assayed for $^{14}CO_2$ production from ^{14}C-labeled 2,4-D as described previously (Amy et al. 1985). The same assay was used on plant material, but with two modifications: the use of plant protoplasts, and the addition of excess unlabelled glyoxylate as a competitive substrate for glyoxylate metabolism. Leaf mesophyll protoplasts were prepared as described (Takebe et al. 1971). A 10-ml aliquot of protoplasts at a density of 1×10^5/ml was incubated in culture medium with 1 ml of a 20 mg/ml glyoxylate solution and 2 μl of ^{14}C-labeled 2,4-D (1.8 μmoles, 0.1 μCi). After 16 h of incubation in the dark, the amount of acid-hydrolyzed $^{14}CO_2$ released from the culture was measured as in the bacterial assay.

Assay of explants for 2,4-D tolerance

Tobacco leaf slices were placed on shoot-induction medium in the absence of 2,4-D or in the presence of 2 μg/ml (10^{-5} *M*) to 6 μg/ml of 2,4-D. The ability to form shoots was scored after 2 weeks. Regenerated cotton plants were screened for resistance to 2,4-D by placing axenic shoot tips into rooting medium containing 0.05 μg/ml 2,4-D or by treatment of the shoot tip with 0.01 μg/ml 2,4-D. Root and new leaf morphologies were observed after 1 week.

Spray-test of greenhouse-grown plants

Tobacco plants were grown in the greenhouse to a height of 6 inches. Each plant was sprayed with approximately 10 ml of either 0.2%, 0.5% or 1.0% 2,4-D solution (pH 5.6) or with water as a control. The area covered by each spraying was approximately 500 cm^2, equivalent to 2,4-D dosages of 4, 10 and 20 kg/ha. Plant growth was observed 3 weeks after spraying. For cotton, primary transformants that were resistant to the root or meristem 2,4-D treatment were transferred to the greenhouse, grown to a height of approximately 8 inches and then sprayed with a range of 2,4-D concentrations (0, 10, 300, 1,000 and 3,000 mg/l) using a spray chamber designed to simulate field spray conditions. The spray pattern covered 3.7 ft^2 and at 3,000 ppm delivered the equivalent of 1.5 kg/ha of 2,4-D. Testing for herbicide resistance on the progeny from a transformed plant was conducted at the two-leaf stage using a 1.5 kg/ha commercial preparation of 2,4-D, and the plants were monitored for damage over a period of several weeks.

Molecular analysis of plant DNA

Plant genomic DNA was extracted from young leaves and PCR analysis (Saiki et al. 1988) was performed using two 20-bp primers: 5′-CACAATCCCACTATCCTTCG-3′ (35S promoter sequence) and 5′-GACGGCATCGTCCAGGGTGG-3′ (*tfd*A gene sequence). Approximately 1 μg of template DNA, 0.25 μg of each primer and 0.2 m*M* dNTP were used along with Taq polymerase and the buffer solution as recommended by the manufacturer (Promega). The PCR profile used was 94 °C, 1 min, 55 °C, 2 min, 72 °C, 2 min for a total of 30 cycles on a Perkin-Elmer Cetus DNA thermal cycler.

Results and discussion

Isolation of the 2,4-D monooxygenase (tfdA) gene from A. eutrophus plasmid pJP5

Genomic DNA from *A. eutrophus* and *E. coli* strains harboring pJP5 were screened for homology using synthetic oligonucleotides corresponding to the sequence of the pJP4-derived *tfd*A gene (Streber et al. 1987). A XbaI to SalI fragment of approximately 2 kg was detected from both strains by Southern hybridization. Gel-purified XbaI/SalI-cleaved DNA from *E. coli* (pJP5) was inserted into pUC19 to obtain a partial genomic library, and colony hybridization was used to identify plasmids containing DNA homologous to the pJP4-derived *tfd*A sequence. Homology to *tfd*A of pJP4 was confirmed by sequencing the 5′ and 3′ ends of the putative coding region.

As in the case of pJP4, GTG appeared to be the translation start codon of the *tfd*A gene in pJP5. For translation in plant cells, part of the 5′ coding region was removed and replaced with a synthetic oligonucleotide containing an ATG start codon (Fig. 1). The modified gene was inserted between the cauliflower mosaic virus 35S promoter (35S) and the polyadenylation signal of the

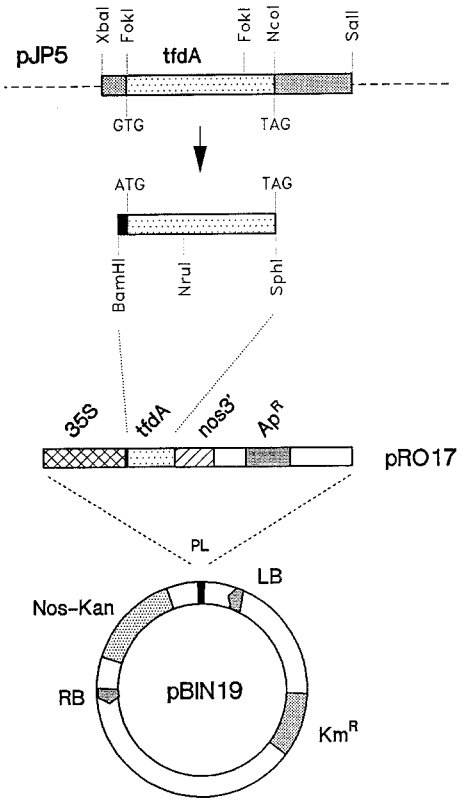

Fig. 1. Schematic representation of the *tfd*A gene from pJP5 leading to constructs pRO17 and pBIN19 : : pRO17. Only restriction sites relevant to the text are shown. *35S* cauliflower mosaic virus 35S promoter, *nos3'* polyadenylation signal-containing fragment from nopaline synthase gene, Ap^R bacterial ampicillin resistance gene, Km^R bacterial kanamycin resistance gene, *Nos-Kan* nopaline synthase promoter-kanamycin chimeric gene which confers resistance to kanamycin to plant cells, *RB* T-DNA right border sequence, *LB* T-DNA left border sequence, *PL* polylinker cloning site

nopaline synthase gene (*nos*3') to produce pRO17 (Fig. 1). Since the CaMV 35S promoter has activity in bacteria (Assaad and Signer 1990), pRO17 was tested for *tfd*A expression in *E. coli* prior to introducing it into plants. Activity of 2,4-D monooxygenase in *E. coli* was determined by measuring the conversion of ^{14}C-labeled 2,4-D into $^{14}CO_2$ (Amy et al. 1985). As with *A. eutrophus* harboring pJP5, *E. coli* carrying pRO17 was efficient in releasing $^{14}CO_2$, showing that the modified *tfd*A gene in pRO17 encodes functional 2,4-D monooxygenase (Table 1).

Transgenic tobacco plants

Since tobacco can be transformed readily, it was used to evaluate the effectiveness of this chimeric construct. pRO17 was transferred into *Agrobacterium tumefaciens* as a cointegrate plasmid with pBIN19, a vector used for plant transformation (Bevan 1984) (Fig. 1). Kanamycin-resistant plants were obtained following *Agrobacterium* infection of tobacco leaves. Leaves from putative transformed plants were placed on shoot-regeneration medium containing 2 mg/l, 4 mg/l and 6 mg/l 2,4-D. Seven transformants were able to produce shoots at 6 mg/l 2,4-D and two others at 4 mg/l 2,4-D. In contrast, nontransformed tobacco leaves were unable to produce shoots at 2 mg/l 2,4-D. Vegetatively derived clones of these primary transformants were transferred to the greenhouse and sprayed with varying doses of 2,4-D. Whereas non-transformed plants were sensitive at 4 kg/ha, one line was resistant to 10 kg/ha and seven lines were unaffected at levels up to 20 kg/ha (Fig. 2).

As evidence that the mechanism of 2,4-D resistance in the transgenic plants was due to degradation of the herbicide, $^{14}CO_2$ released from acid hydrolysis of glyoxylate, an enzymatic product of 2,4-D monooxygenase action on ^{14}C-labeled 2,4-D, was measured. Since plant cells can metabolize glyoxylate, excess unlabeled glyoxylate was supplemented to the incubation solution to reduce metabolism of the ^{14}C-labeled product. In a sampling of six lines of primary tobacco transformants, $^{14}CO_2$ production was 11- to 85-fold higher than that

Table 1. 2,4-D monooxygenase activity in (A) bacteria, (B) tobacco and (C) cotton. For tobacco, leaf mesophyll protoplasts from primary transformants were used. For cotton, protoplasts from cotyledons and true leaves were isolated from two seedlings derived from self-pollination of plants 2, 3 and 14 and previously determined by PCR analysis to harbor the *tfd*A gene. Activity is defined as counts per minute (cpm) of $^{14}CO_2$ released after incubation in medium containing ^{14}C-labeled 2,4-D

	$^{14}CO_2$ (10^3 cpm)	Relative activity
(A) Bacteria		
E. coli	2.7	1
E. coli (pRO17)	198	73
A. eutrophus (pJP5)	111	41
(B) Tobacco		
Nontransformed control	0.15	1
Transformant 3	1.6	11
Transformant 4	10.4	69
Transformant 5	5.3	35
Transformant 7	4.3	29
Transformant 12	10.4	69
Transformant 13	12.7	85
(C) Cotton		
Nontransformed control	0.6	1
Transformant 2.1	22.1	37
Transformant 2.6	22.7	38
Transformant 3.1	9.9	17
Transformant 3.2	14.3	24
Transformant 14.1	21.8	36
Transformant 14.4	21.1	35

Fig. 2. Comparison of transgenic and wild-type tobacco plants sprayed with 2,4-D. Two wild-type plants (*left*) and two clonal replicas (*right*) of a representative *tfd*A transgenic line (no. 4) are shown 3 weeks after treatment with 2,4-D equivalent to 20 kg/ha

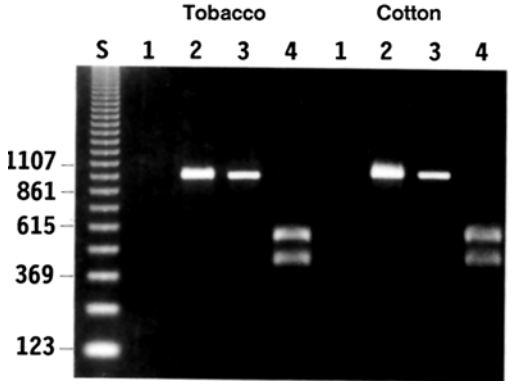

Fig. 3. PCR analysis of tobacco and cotton plants. PCR reactions examined for the presence of the predicted 940-bp *tfd*A-specific sequence in a non-transformed and a representative transformant are shown in *lanes 1* and *2*, respectively. *Lanes 3* and *4* show, respectively, no treatment and treatment with endonuclease NruI of the 940-bp PCR products purified from reactions on the same transformed tobacco or cotton DNA. NruI cleavage of the 940-bp product yielded two new fragments of the expected sizes of about 500 bp and 400 bp. Shown in lane *S* are size markers (BRL 123-bp DNA ladder), and relevant sizes (in bp) are as indicated

from non-transformed material (Table 1), indicating that resistance to 2,4-D was due to herbicide degradation.

In addition to enzyme activity, polymerase chain reaction (PCR) analysis was used to detect the presence of the 35S-*tfd*A gene fusion. Primers that anneal to the 35S promoter and the carboxyl end of the *tfd*A gene were used to amplify a fragment with an expected size of 940 bp. This 940-bp PCR fragment was indeed cleaved by endonuclease NruI to yield two fragments of approximately the expected sizes of 513 bp and 427 bp (Fig. 3). Seeds of two transgenic lines were examined in a germination assay. Whereas all 112 non-transformed seeds germinated only at 10^{-7} *M* 2,4-D, seeds derived from primary transformants numbers 3 and 4 germinated in 10^{-3} *M* 2,4-D with frequencies of 77% (62/80) and 74% (73/99), respectively. These 3 to 1 segregation ratios suggested that both primary transformants harbor a single 2,4-D resistance locus.

Transgenic cotton plants

The central focus of this work was to engineer 2,4-D tolerance into cotton. The same chimeric *tfd*A gene in pBIN19 : : pRO17 was therefore introduced into cotton by *Agrobacterium*-mediated transformation. Shoots of putative transformed plants were placed on rooting medium containing 0.05 mg/l 2,4-D and/or meristems were treated with 0.01 mg/l 2,4-D. Approximately 300 transformants from 30 cell lines rooted normally and/or were unaffected by the meristem treatment. Vegetatively derived clones of the primary transformants were transferred to the greenhouse and sprayed with varying levels of 2,4-D. Transformants were unaffected up to 3 times the recommended spray rate of 2,4-D, whereas non-transformed plants were severely affected at 300 times less the recommended spray rate of the herbicide. Higher levels of 2,4-D were not tested.

Progeny derived from self-fertilization of three transformed lines (numbers 2, 3, 14) were analyzed further. PCR analysis confirmed the presence of the *tdf*A gene in representative progeny seedlings (Fig. 3). Two *tfd*A transgenic seedlings from each line, as shown by PCR analysis, were assayed for 2,4-D monooxygenase. Activity 17- to 38-fold above that of the non-transformed control was found from the *tfd*A-containing plants (Table 1). In a germination assay, progeny from wild-type plants were unable to form roots on medium containing 0.1 mg/l 2,4-D. In contrast, nearly three-quarters of the germinating progeny seedlings from each of the three transformed lines were unaffected by the herbicide. The 3 : 1 segregation pattern is indicative of a single 2,4-D resistance locus. Direct spraying of the herbicide was conducted on the progeny of primary transformant no. 2. Plants germinated in the absence of 2,4-D were sprayed at the two-leaf stage with a commercial preparation of the herbicide set at a dosage equivalent of 1.5 kg/ha (Fig. 4). This is approximately 3 times the manufacturer's highest recommended rate for wheat, corn, sorghum and pastures (0.2, 0.2, 0.2 and 0.6 kg/ha, respectively). Whereas all 18 wild-type plants were killed within 5 days, 83 out of 107 progeny plants derived from transformant no. 2 were unaffected by the herbicide treatment.

Concluding remarks

Our overall goal was to reduce annual losses of cotton due to drift-levels of 2,4-D. Towards this aim, we isolated and modified a bacterial gene encoding 2,4-D monooxygenase and tested its effectiveness in tobacco. The result-

Fig. 4. Comparison of wild-type and transgenic cotton plants 3 weeks after treatment with a commercial preparation of 2,4-D: wild-type cotton sprayed with 0.15 kg/ha 2,4-D (*leftmost*), and representative progeny plants from transgenic line no. 2 treated with 0.15 kg/ha (*second from left*), 0.5 kg/ha (*second from right*) and 1.5 kg/ha of the herbicide (*rightmost*)

ing transgenic tobacco plants produced functional 2,4-D monooxygenase and were resistant to the herbicide. In previous reports, Streber and Willmitzer (1989) and Lyon et al. (1989) also obtained 2,4-D-resistant tobacco plants using a *tfd*A gene isolated from a different *A. eutrophus* plasmid. Transgenic tobacco plants were found to be resistant to 2,4-D at 10 kg/ha and 6 kg/ha, respectively, which are in the same range of resistance as the tobacco plants described here. In the case of cotton, tolerance to at least 1.5 kg/ha was found, which is 3 times the highest recommended level used for wheat, corn, sorghum and pasture. Moreover, this level of resistance was achieved using commercial preparations of the herbicide that contain emulsifiers and surfactants. The degree of tolerance achieved should be more than adequate to protect cotton from drift-levels of the herbicide.

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References

Amy PS, Schulke JW, Frazier LM, Seidler RJ (1985) Characterization of aquatic bacteria and cloning of genes specifying partial degradation of 2,4-Dichlorophenoxyacetic acid. Appl Environ Microbiol 49:1237–1245

Assaad FF, Signer ER (1990) Cauliflower mosaic virus P35S promoter activity in *Escherichia coli*. Mol Gen Genet 223:517–520

Bevan M (1984) Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res 12:8711–8721

Don RH, Pemberton JM (1981) Properties of six pesticide degradation plasmids isolated from *Alcaligenes paradoxus* and *Alcaligenes eutrophus*. J Bacteriol 145:681–686

Horsch RB, Fry JE, Hoffman NL, Eichholtz D, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. Science 227:1229–1231

Lyon BR, Llewellyn DJ, Huppatz JL, Dennis ES, Peacock WJ (1989) Expression of a bacterial gene in transgenic tobacco plants confers resistance to the herbicide 2,4-dichlorophenoxyacetic acid. Plant Mol Biol 13:533–540

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Ow DW, Wood KV, DeLuca M, deWet JR, Helinski DR, Howell SH (1986) Transient and stable expression of the firefly luciferase gene in plant cells and transgenic plants. Science 234:856–859

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) Primer directed amplication of DNA with a thermostable DNA polymerase. Science 239:487–491

Stewart J McD, Hsu CL (1977) In ovulo embryo culture and seedling development of cotton (*Gossypium hirsutum* L.). Planta 137:113–117

Streber WR, Willmitzer L (1989) Transgenic tobacco plants expressing a bacterial detoxifying enzyme are resistant to 2,4-D. Biotechnology 7:811–816

Streber WR, Timmis KN, Zenk MH (1987) Analysis, cloning, and high-level expression of 2,4-dichlorophenoxyacetate monooxygenase gene *tfd*A of *Alcaligenes eutrophus* JMP134. J Bacteriol 169:2950–2955

Takebe I, Labib G, Melchers G (1971) Regeneration of whole plants from isolated mesophyll protoplasts of tobacco. Naturwissenschaften 88:318–320

Trolinder N, Goodin JR (1987) Somatic embryogenesis and plant regeneration in cotton (*Gossypium hirsutum*). Plant Cell Reports 6:231–234

Umbeck P, Johnson G, Barton K, Swain W (1987) Genetically transformed cotton (*Gossypium hirsutum* L.) plants. Bio/Technology 5:263–266