

TRANSGENIC TOBACCO PLANTS EXPRESSING A BACTERIAL DETOXIFYING ENZYME ARE RESISTANT TO 2,4-D

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We have used a gene from the soil bacterium *Alcaligenes eutrophus* JMP134 to introduce a herbicide degrading mechanism into plants. The gene, *tfdA*, which encodes a 2,4-dichlorophenoxyacetate monooxygenase (DPAM) catalyzing the first step in the bacterial 2,4-D degradative pathway, has recently been cloned and sequenced. The coding sequence, fused with plant expression sequences was introduced into plants via *Agrobacterium*-mediated gene transfer. The chimeric gene was expressed in tobacco (*Nicotiana tabacum*) under the control of either a constitutive or a light-inducible promoter. Transgenic tobacco lines tolerated elevated levels of 2,4-D in tissue culture, and regenerated plants showed resistance when sprayed with the herbicide.

Phenoxycetic acids, such as 2,4-D and MCPA (4-chloro-2-methylphenoxyacetic acid) show an auxin-like hormone action when applied to plants. It is postulated they mimic the function of the intrinsic auxin, indole-3-acetic acid, on an internal auxin receptor of the plant. The fact that higher concentrations of the synthetic auxins are toxic to many dicotyledonous, but not to monocotyledonous, plants, has been widely used for their application as herbicides with high selectivity to control broadleaf weeds in cereal and grass crops.

Considerable effort has been spent to create herbicide resistant crop plants. Although various attempts to isolate such mutants or variants by conventional breeding and tissue culture methods have met so far with limited success, the methods of genetic engineering opened up the way to transfer effective resistance mechanisms to plants¹. Resistance to atrazine², glyphosate³, and sulfonylurea herbicides⁴ have been achieved by the introduction of foreign genes encoding modified insensitive target proteins. Resistance to phosphinotricin⁵ and bromoxynil⁶ were based on the expression of detoxifying enzymes originally isolated from microorganisms.

Attention has also been given to the possibility of generating dicotyledonous plants resistant to 2,4-D. In addition to its agricultural value, the study of 2,4-D resistant plants may improve our knowledge about the site of action and the molecular mechanisms by which auxins exert their effects. 2,4-D-resistant mutants have been isolated after mutagenesis of *Arabidopsis thaliana*⁷. Similar to 2,4-D resistant tobacco cell lines⁸, these mutants showed a severely altered phenotype. Genetic and biochemical studies suggested that resistance was due to a mutation

affecting a function involved in the action and not in the metabolism of 2,4-D.

A potentially useful approach to the production of 2,4-D resistant plants that show a normal phenotype would therefore be to introduce a gene into plant cells known to encode the metabolism of 2,4-D into a less toxic compound. A gene fulfilling these needs has recently been cloned and sequenced⁹. The *tfdA* gene from the soil bacterium *Alcaligenes eutrophus* JMP134 encodes the first enzyme involved in a 2,4-D degradative pathway: A single polypeptide that is able to remove the acetate side chain from 2,4-D in a monooxygenase-like reaction.

It was not known if the bacterial degradative monooxygenase would be enzymatically active in plants, or if its broad substrate specificity would interfere with the plant metabolism. Here we report the expression of *tfdA*, encoding a 2,4-dichlorophenoxyacetate monooxygenase (DPAM) in genetically engineered tobacco plants. We show that transgenic plants that express DPAM are resistant to the herbicide 2,4-D, and we describe the phenotypic response of the transgenic plants to differently substituted phenoxy herbicides in tissue culture.

RESULTS

Construction of plasmids for constitutive and light-induced DPAM expression in plants. To create a plant/bacterial hybrid gene for DPAM the prokaryotic translation initiation codon GTG of *tfdA* was replaced by an ATG codon. This was achieved by a synthetic double stranded oligonucleotide designed to fit a 5' protruding end generated by FokI 8 bp downstream from the translation initiation codon. The oligonucleotide contained a sequence reconstituting the original open reading frame for DPAM, the consensus sequence for plant translational start sites upstream from the ATG start codon¹⁰ and a 5' protruding end compatible with BamHI cloning sites. The oligonucleotide was linked with the coding sequence of *tfdA* truncated at its N-terminal end by a partial digest with FokI (Fig. 1). The correct ligation of the oligonucleotide was verified by sequence analysis.

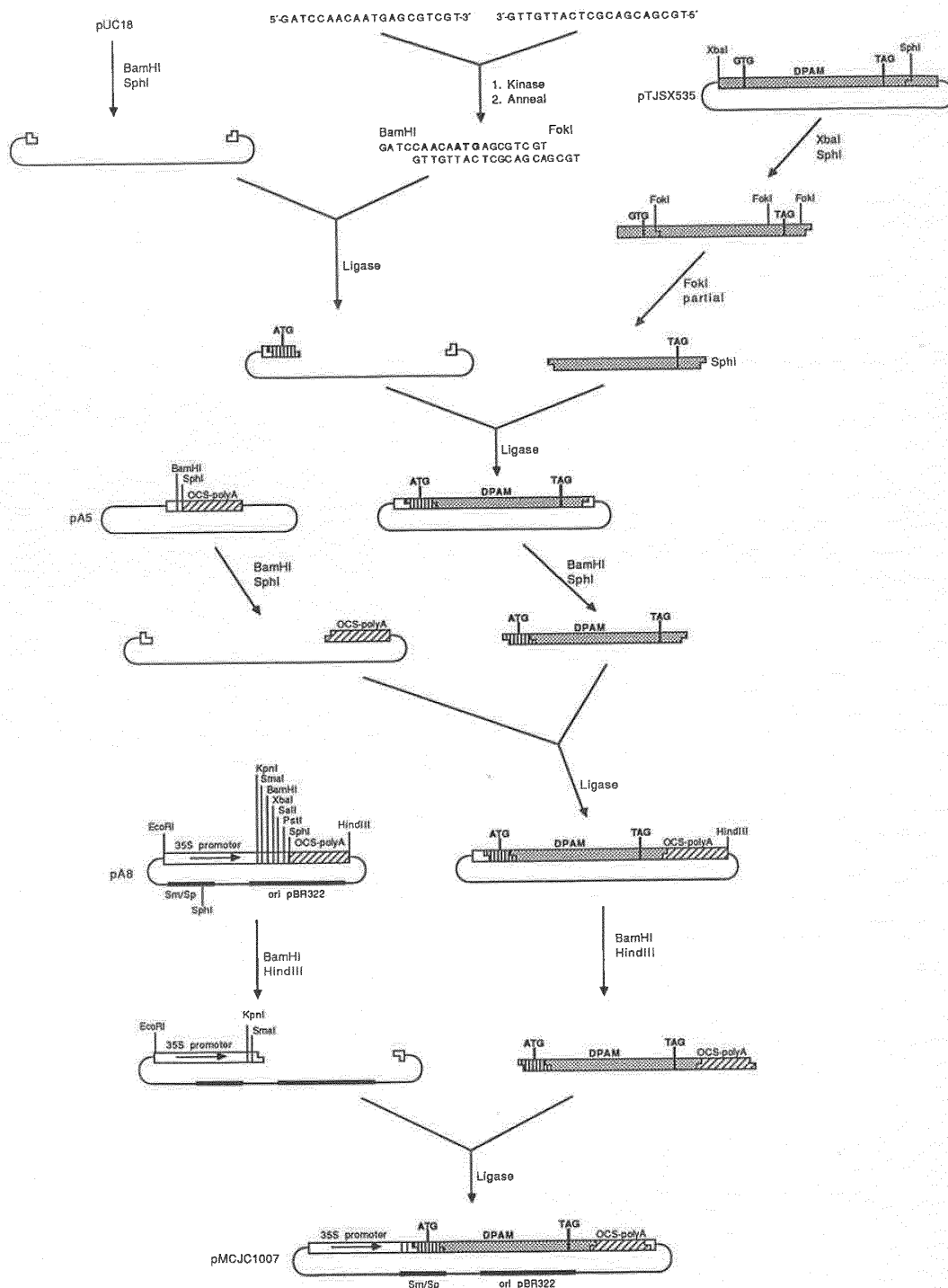
The new sequence was fused at its 3' end to a 200 bp fragment containing the polyadenylation signal of the octopine synthase gene (OCS-polyA)¹¹ and at its 5' end to a 540 bp fragment of the cauliflower mosaic virus (CaMV) 35S promoter^{12,13}. The resulting plasmid, designated pMCJC1007 (Fig. 1), was based on the intermediate vector pMPK110 which is able to form cointegrates with disarmed Ti-plasmids of *A. tumefaciens* such as pGV3850kan¹⁴. In a similar construction the constitutive CaMV 35S promoter was replaced by the light-inducible promoter of gene ST-LS1¹⁵ to form a plasmid designated pMLJC1005 (data not shown).

Toxicity of the DPAM reaction product. Gas chromatography and mass spectrometry of *E. coli* pUJC1001 expressing a DPAM-fusion gene showed that 2,4-dichlorophenol (2,4-DCP) was the reaction product thus confirming previous indirect results⁹.

In order to evaluate the possible toxicity of 2,4-DCP to

FIGURE 1 Construction of plasmid pMCJC1007 for stable integration and expression of the DPAM gene in plants. In a first cloning step the procaryotic translational start codon GTG of gene *tda* was replaced by a synthetic oligonucleotide. To avoid dimerisation at the BamHI site the oligonucleotide was linked in a two step ligation first to the BamHI end of linearised pUC18 and then to the DPAM coding sequence. In

two following cloning steps, the BamHI-SphI fragment containing the hybrid construct was inserted into the plant expression vector plasmid pA8, between the CaMV 35S promoter and the OCS 3' end. As pA8 contains a second SphI site outside of its polylinker, pA5 was used to form an intermediate plasmid based on pUC18.



plant cells, callus cultures of tobacco were placed onto MS-agar plates containing 1 mg/l NAA, 0.1 mg/l BAP and 2,4-DCP in concentrations ranging from 1 to 10 mg/l. No toxic effects could be seen with 10 mg/l 2,4-DCP, whereas more than 2 mg/l 2,4-D severely reduced growth and turned calli brown. Perkins *et al.*¹⁶ reported 50% growth inhibition of tobacco suspension cultures by 10 mg/l 2,4-DCP, but a generally much lower toxicity of 2,4-DCP on the development of leaves and roots when compared to 2,4-D. These results were encouraging with respect to the idea that transformation of tobacco with the DPAM gene could result in resistance to 2,4-D.

Transformation of tobacco with DPAM genes and transcript analysis. Both constructs containing the hybrid DPAM genes in combination either with the constitutive (pMCJC1007) or with the light inducible promoter (pMLJC1005) were used to transform tobacco (*Nicotiana tabacum* W38). Gene transfer was mediated by *Agrobacterium* strain C58C1 carrying the disarmed Ti-plasmid pGV3850kan¹⁴ that is able to form cointegrates with pBR322 derived vectors. Shoots were regenerated from infected leaf discs by selection on kanamycin, screened for nopaline production and later analyzed by Northern blot hybridization for the synthesis of DPAM-RNA. Twenty out of 25 transformed plants synthesized a specifically hybridizing transcript of the expected size of 1.3 kb (data not shown).

Expression of the DPAM gene in tissue culture of tobacco. 2,4-D is known to promote callus growth and to suppress shoot regeneration from explanted tobacco leaf discs in tissue culture when applied in concentrations higher than 0.1 mg/l. To test transgenic plants for their ability to tolerate elevated levels of 2,4-D, we chose a "shoot regeneration" medium containing 1.0 mg/l BAP and 2,4-D at concentrations of 0.1, 0.2, 0.4, 1.0, 2.0, and 4.0 mg/l. Leaf discs from tobacco plants where the DPAM gene was transcribed from the constitutive 35S promoter were able to develop normal shoots on a medium containing 1.0 mg/l 2,4-D (Fig. 2A). Depending on the individual transformed plant tested, minimal shoot inhibition is observed at concentrations of 2.0 or 4.0 mg/l 2,4-D, whereas leaf discs from untransformed control plants (tobacco W38) did not show any differentiation on 0.2 mg/l 2,4-D, indicating that expression of the DPAM gene could result in up to 20-fold higher tolerance against 2,4-D.

A significantly different reaction was shown by plants where the DPAM gene is under the control of the light-inducible promoter of the ST-LS1 gene. As this promoter is only active in photosynthetically active tissues, and shoot differentiation from explanted tobacco leaf discs proceeds via a (photosynthetically not active) callus stage, as expected in the shoot suppression assay, the transgenic DPAM-plants showed the same sensitivity against 2,4-D as the nontransformed tobacco W38 (Fig. 2B).

Response to differently substituted phenoxyacetic acids. In *Alcaligenes eutrophus* JMP134 from which the DPAM gene was originally isolated, DPAM is able to degrade 2,4-D, MCPA, 4-CPA and unsubstituted phenoxyacetic acid. To investigate how transgenic tobacco expressing DPAM responds to the auxin activity of differently substituted phenoxyacetic acids, the substances listed in Figure 3 were incorporated in MS-agar together with 1.0 mg/l BAP. Leaf discs of transgenic tobacco WS7-7 and control W38 were placed on the media and shoots arising were counted after 18 days. Figure 3 shows, that transgenic plants expressed a similar tolerance to 4-CPA as to 2,4-D, a slightly reduced tolerance to MCPA and no tolerance against tri-substituted phenoxyacetic acids and all the phenoxypropionic acids we tested.

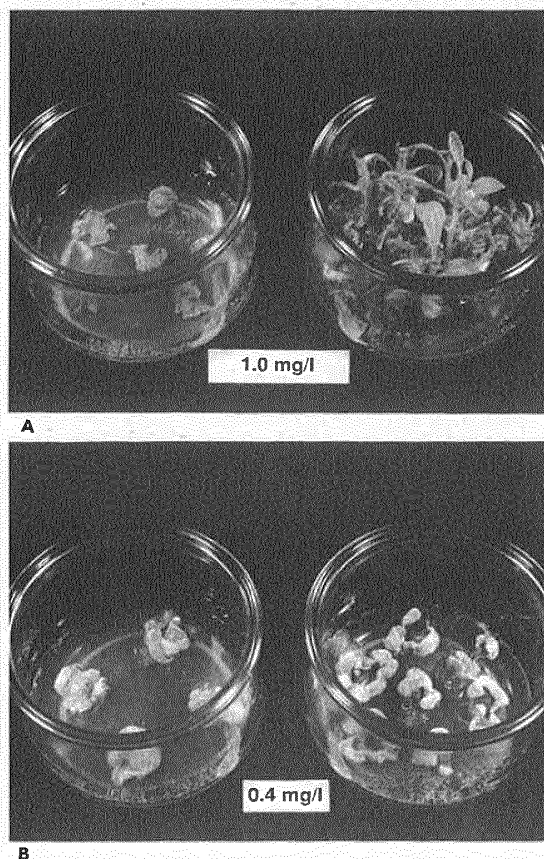


FIGURE 2 Tolerance of transgenic tobacco against 2,4-D in tissue culture. Explants from *Nicotiana tabacum* W38 control plants (left) and transgenic plants (right) were incubated on medium containing 1.0 mg/l 2,4-D. Normal shoots develop from leaf discs which constitutively express DPAM (A, right). Transgenic explants with a light-inducible DPAM gene (B, right) show the same sensitivity against 2,4-D as the nontransformed tobacco W38 (left).

Transgenic plants are resistant to the herbicidal activity of 2,4-D. Replicates of transgenic plants obtained by clonal propagation were transferred to soil and grown in the greenhouse. No difference in growth behaviour and final height could be seen between untransformed and transformed tobacco. After two weeks of growth in soil control tobacco plants and transgenic tobacco plants were sprayed with doses equivalent to 1 kg/ha, 3 kg/ha and 10 kg/ha 2,4-D-Na. Plants were further grown for 2-3 weeks in the greenhouse and then checked for resistance. Whereas 1 kg/ha totally inhibited growth of nontransformed tobacco plants, plants of the transgenic line WS7-7 which expressed the DPAM gene under the control of the constitutive 35S promoter were resistant to up to 10 kg/ha 2,4-D-Na (Fig. 4 A).

Plants of the transgenic line WS4-7 that expressed the DPAM gene under a light-inducible promoter active only in green tissue and thus did not show tolerance in a tissue culture assay, were also resistant to the herbicide when sprayed with 1 kg/ha 2,4-D-Na (Fig. 4 B). No difference in growth behaviour and final height was observed between transgenic plants treated with the herbicide and non-treated plants (Fig. 4 C).

Inheritance of 2,4-D resistance. Seeds from selfed WS7-7 plants were germinated on sterile MS medium with kanamycin. Two hundred and fifty eight seedlings were resistant to kanamycin and 83 were sensitive indicating Mendelian inheritance of a single active locus. Leaf segments from 16 two weeks old Km^r F1 plants were placed on 2,4-D medium to check for 2,4-D resistance, another 20 plants were sprayed with 3 kg/ha 2,4-D-Na. In all cases inheritance of 2,4-D resistance was observed. Southern blots showed that the parental line WS7-7 contained at least three copies of the DPAM gene, which could also be found in 14 out of 15 analysed transgenic F1 plants (data not shown). This suggests that the three T-DNA copies in line WS7-7 were linked.

DPAM as a selectable marker. The use of the constitutively expressed DPAM gene as a selectable marker for *Agrobacterium* mediated leaf disc transformation was examined. In a standard transformation procedure, kanamycin was replaced by 2,4-D at a concentration of 0.5 mg/l, which was previously shown to be appropriate to distinguish between untransformed and completely transformed tissue, but no shoot development was observed under these conditions.

In order to test if DPAM expression could be used for selection of germinating seeds, root formation from seeds was measured in response to different concentrations of 2,4-D. Inhibition curves (Fig. 5) demonstrate that no significant difference in sensitivity is seen between untransformed seedlings and seedlings containing the 35S-DPAM gene.

DISCUSSION

The *tfdA* gene from *Alcaligenes eutrophus* JMP134 encodes the enzyme 2,4-dichlorophenoxyacetate monooxygenase (DPAM) that degrades the herbicide, 2,4-D. The degradation product, 2,4-dichlorophenol, has been shown to be non-toxic to plants at least up to concentrations of 10 mg/l. We have fused the DPAM coding sequence to both a constitutive and a light-inducible promoter and transferred the constructs in tobacco using an *Agrobacterium* vector system. Transgenic tobacco plants transformed with the chimeric DPAM-genes were fully resistant to high doses of 2,4-D. In addition, leaf cells from transgenic tobacco that constitutively express DPAM are able to redifferentiate into shoots in the presence of concentrations of 2,4-D that normally suppress shoot formation.

Unlike the NPT gene¹⁷ or the PAT gene⁵, the DPAM gene could not be used as a selectable marker in leaf disc transformation. One reason may be that in a chimeric tissue transformed cells cannot develop into shoots because they are overgrown or otherwise inhibited by untransformed callus that is rapidly developing in the presence of 2,4-D. It remains to be examined whether transformation of protoplasts instead of leaf discs might overcome this problem.

It is also not possible to select transgenic DPAM plants during germination on 2,4-D. The lack of resistance of roots against 2,4-D may have several reasons, e.g. a lower expression of the 35S promoter in roots, a higher sensitivity of roots against 2,4-D or 2,4-DCP compared to shoots or an accumulation of 2,4-D or 2,4-DCP by transport.

The analysis of the DPAM-specific mRNA demonstrated a substantial variation in expression levels of around 20 to 30-fold between independent transformants. Nevertheless, the highest expression level of the DPAM gene in line WS7-7 corresponded to only three T-DNA copies in the genome (data not shown). A similar observation has been made by DeBlock et al.⁵ who reported three copies of the PAT gene for the highest expressing plant. He suggested that the observed 30-fold differences in the expression of

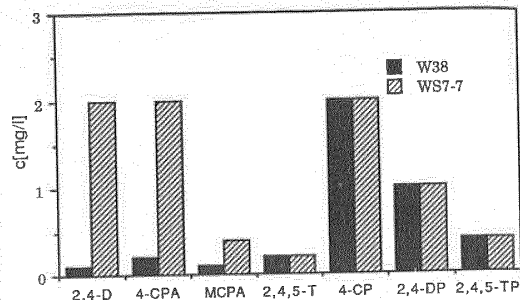


FIGURE 3 Cross tolerance of transgenic tobacco against other phenoxy herbicides. Substances listed below were incorporated into 2MS agar as described in the text. The graph demonstrates the response of control tobacco (W38, black columns) and transgenic tobacco (WS7-7, hatched columns) against different synthetic auxins. The height of the columns indicates the upper limit that allowed cells from explanted leaf discs to differentiate into normal shoots. At higher concentration only callus growth was observed. 2,4-D: 2,4-dichlorophenoxyacetic acid, 4-CPA: 4-chlorophenoxyacetic acid, MCPA: 4-chloro-2-methyl-phenoxyacetic acid, 2,4,5-T: 2,4,5-trichlorophenoxyacetic acid, 4-CP: 2-(4-chlorophenoxy)propionic acid, 2,4-DP: 2-(2,4-dichlorophenoxy)propionic acid, 2,4,5-TP: 2-(2,4,5-trichlorophenoxy)propionic acid.

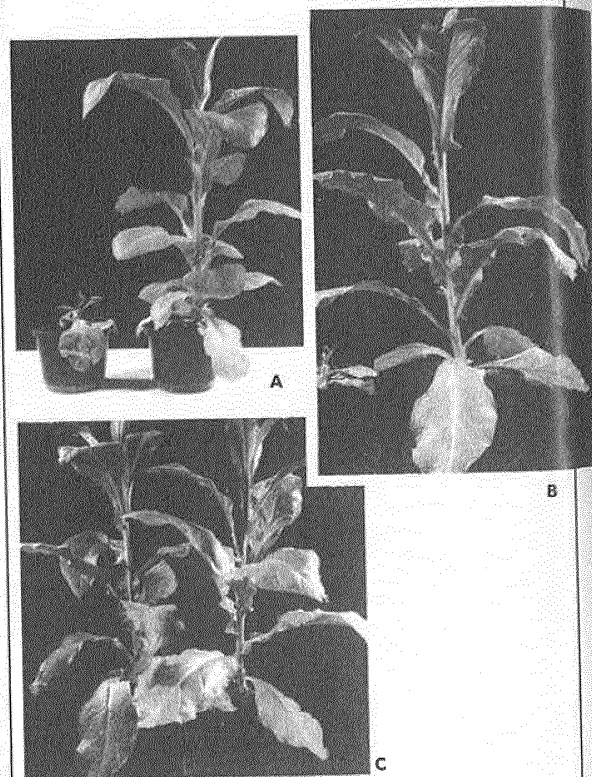


FIGURE 4 Effect of 2,4-D on transgenic tobacco plants. A. A *Nicotiana tabacum* W38 control plant (left) and a clonal replicate of the WS7-7 line that expresses the DPAM gene under the control of the CaMV 35S promoter (right) were sprayed with 10 kg/ha 2,4-D-Na. B. A *Nicotiana tabacum* W38 control plant (left) and a clonal replicate of the WS4-7 line that expresses the DPAM gene under the control of the light-inducible ST-LS1 promoter (right) were sprayed with 1 kg/ha 2,4-D-Na. C. Two replicates of the WS4-7 line were sprayed either with water (left) or with 1 kg/ha 2,4-D-Na (right). The pictures were taken three weeks after spraying.

PAT-RNA may be due to effects of the T-DNA position in the chromosome.

Expression of DPAM-RNA in transformants where the DPAM gene is under the control of the light-inducible promoter varied in the same range, but we never observed similarly high expressing variants compared to the constitutively expressing plants. The highest observed expression corresponded to a resistance against sprayed doses of 1 kg/ha 2,4-D-Na. No qualitative difference could be seen between resistance phenotypes generated by the constitutive or the light-inducible DPAM gene.

When transgenic plants expressing DPAM were sprayed with 2,4-D in the greenhouse, they were fully resistant to amounts of 2,4-D commercially applied in the field. For example, line WS4-7 tolerates 1 kg/ha, line WS7-7 tolerates 10 kg/ha 2,4-D-Na. Depending on the crop and weed species, 2,4-D is effectively applied in amounts ranging between 0.3 kg/ha and 3 kg/ha. This result is encouraging with respect to the application of the DPAM gene to create 2,4-D resistance in other commercially interesting crop plants. As 2,4-D is already a selective herbicide in monocotyledonous crop cultures, dicotyledonous crop plants will be the preferred targets for genetic engineering. Additional investigation of the fate of the DPAM reaction product, 2,4-dichlorophenol, in the plant are required. It may be worthwhile to think about introducing further genes of the bacterial 2,4-D degradative pathway into plants. In any case, toxicology and persistence of 2,4-D and its degradation products have to be newly evaluated.

Other attempts to obtain 2,4-D resistant plants have been made^{7,18}. The introduction of the detoxifying enzyme DPAM by genetic engineering is up to now the only approach that results in fully resistant and phenotypically normal plants. This strategy promises to have broad application, especially with herbicides for which detoxifying pathways can be found in nature or created by genetically modifying existing pathways. Future development of new herbicidal compounds should favour substances that are easily degraded by microorganisms, thus providing environmentally safe, non-persisting herbicides together with the opportunity to introduce these selective resistances into crop plants by genetic engineering.

EXPERIMENTAL PROTOCOL

Strains. *E. coli* TB1 is a derivative of strain JM101¹⁹ with the genotype: F'(traD36, proAB, lacI, lacZΔM15) Δ(lac, pro), supE, thi, recA, Sfi1::Tn10(Tc') (Bart Barrel, pers. communication). *E. coli* GJ23²⁰ was used as a helper strain for triparental matings. *Agrobacterium tumefaciens* strain C58C1 (pGV3850kan)¹⁴ contains a disarmed co-integrate-type Ti-plasmid.

Plasmids. pUC18 was described by Norrander²¹. pA5 (Fig. 1) contains 200 bp of the octopine synthase 3'-end¹¹ inserted into pUC18 (A. von Schaewen, in preparation). pA8 (Fig. 1) is a plant expression vector constructed by insertion of the CaMV 35S promoter¹² and the OCS-3' end¹¹ into pMPK110 (A. von Schaewen, in preparation). The multiple cloning site of pA8 was derived from pUC18. pTJSX535 contains the coding sequence of *tfdA* from *Alcaligenes eutrophus* JMP134 on a 1.5 kb XbaI-SalI fragment⁹.

Media. Synthetic media for plant transformation and shoot regeneration from transformed and untransformed tissue were based on MS medium²² containing 20 g/l sucrose. Basic chemicals and 2,4-D were from Merck, Darmstadt, FRG. All other synthetic hormones were from Sigma, St. Louis, MO, USA.

Synthetic oligonucleotides. Oligonucleotides were synthesized by an Applied Biosystems 380A equipment and purified by preparative polyacrylamide gel electrophoresis as described in the Applied Biosystems User Bulletin issue No. 13, 1984 (Applied Biosystems, Foster City, California).

Plasmid constructions. Pairs of complementary oligonucleotides, each consisting of 20 bases, were phosphorylated by polynucleotide kinase, annealed by shifting temperature from 60°C to 37°C, and then ligated with pUC18 that had been digested with

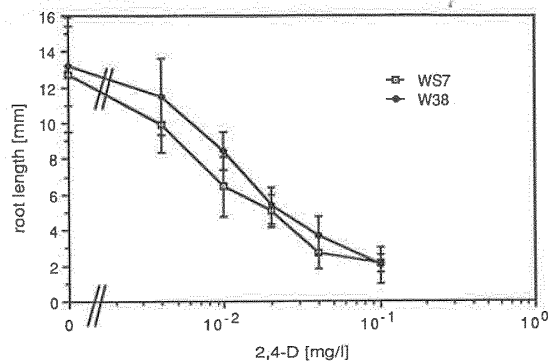


FIGURE 5 Effect of 2,4-D on rooting of seeds. Transgenic tobacco seeds containing the 35S-DPAM gene (WS7) and untransformed seeds (W38) were germinated on sterile MS agar plates containing 2,4-D at concentrations of 0, 4, 10, 20, 40, and 100 µg/l. After 14 days of incubation in the light, root length was measured. Vertical bars indicate the standard error.

BamHI and SphI. The linear product was separated from an excess of oligonucleotides by agarose gel electrophoresis using DEAE membrane NA45 (Schleicher & Schuell). pTJSX535 plasmid DNA was digested with XbaI and SphI and separated by agarose gel electrophoresis. The 1.2 kb fragment containing the DPAM coding sequence was isolated from the gel by electroelution, partially digested by FokI, and reperfired by agarose gel electrophoresis. DNA fragments of around 1.1 kb isolated from the gel were ligated with the linearised pUC18 that contained the oligonucleotide. The resulting plasmid pUJC1001 was cloned in *E. coli* TB1. DNA of pUJC1001 was digested with BamHI and SphI to excise the insert, and with PvuI to avoid religation of the vector, and then ligated with pA5 that had been digested with BamHI and SphI. pA5 was used as an intermediate vector as pA8 contained a second SphI site outside the polylinker. The resulting plasmid pUJA0 was cloned in *E. coli* TB1. DNA of both pUJA01002 and pA8 was digested with BamHI and SphI, mixed and ligated. The resulting plasmid pMCJA1006 was cloned in *E. coli* TB1.

Transformation of tobacco plants with chimeric genes. pMCJC1007 and pMLJC1005 were mobilized from *E. coli* TB1 into the *Agrobacterium* strain C58C1 (pGV3850kan) by a triparental mating²³ using *E. coli* GJ23 as a helper strain. *Agrobacterium* that by Southern hybridization were shown to contain the DPAM gene were subsequently used for leaf disc infection of *Nicotiana tabacum* W38. Transformation and regeneration of tobacco plants followed established procedures²³.

Cloning. Construction of recombinant DNA molecules, transformation into *E. coli*, and analysis of recombinant clones were performed according to established procedures²⁴.

DNA sequencing. DNA was sequenced by the dideoxy method²⁵ using plasmid DNA as a template²⁶.

Hybridization experiments. RNA isolation from leaves and Northern hybridization experiments were performed as described by Jones¹⁴. A BamHI-HindIII fragment from pUJC1001, carrying the DPAM gene, was radioactively labeled and used as a probe to detect DPAM RNA. Plant DNA was isolated essentially as described by Dellaporta²⁷. For Southern hybridization experiments²⁸ plant DNA was cut with HindIII and probed with a cloned fragment of the nopaline synthase gene.

Herbicide application. Each plant was sprayed with 4 ml of a 1% aqueous solution of 2,4-D, Na-salt. With an estimated ground surface of 400 cm² per plant, this roughly corresponds to 10 kg/ha. Lower quantities were achieved by appropriate dilution of the spray solution.

Acknowledgments

We are grateful to C. Recknagel for excellent performance of plant transformation, to R. Breitfeld for propagation of plants, to U. Kutschka for analysis of plants, to A. v. Schaewen for kind gifts of plasmids, and to H.-J. Scheuermann for gas chromatography and mass spectrometry.

Received 18 January 1989; accepted 30 March 1989.

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