

Use of *Alcaligenes eutrophus* as a Source of Genes for 2,4-D Resistance in Plants

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Abstract. The soil bacterium *Alcaligenes eutrophus* is able to degrade phenoxy herbicides such as 2,4-D [(2,4-dichlorophenoxy)acetic acid]. It has been shown that several degradative genes are located on a large plasmid harbored by this organism. We have demonstrated by HPLC and gas chromatography that 2,4-dichlorophenol is a major metabolite resulting from 2,4-D degradation by *A. eutrophus*. A portion of the large plasmid carrying the 2,4-D catabolic genes has been cloned and mapped by restriction endonuclease digestion. Overlapping subclones have been produced and were used in conjugation experiments in order to identify the first gene involved in 2,4-D detoxification. Once sequenced, this gene will be transferred to dicotyledonous plant cells using available vector systems.

Additional index words. 2,4-D monooxygenase gene, plant cell transformation, gas chromatography, 2,4-dichlorophenol toxicity.

INTRODUCTION

Phenoxy herbicides, such as 2,4-D and MCPA [(4-chloro-2-methylphenoxy)acetic acid] show high selectivity between broadleaf and grass species, enabling them to be used in the control of many broadleaf weeds in cereal and grass crops. A substituted benzoic acid such as dicamba (3,6-dichloro-2-methoxybenzoic acid) also shows high selectivity against broadleaf weeds and is used in barley (*Hordeum vulgare* L.), maize (*Zea mays* L.), oats (*Avena sativa* L.), and wheat (*Triticum aestivum* L.).

Considerable attention has been given to the possibility of generating dicotyledonous crop plants resistant to one or several of the herbicides mentioned above. If such plants could be produced, weed control in broadleaf crops would be facilitated and the effects of contamination by spraying neighboring fields would be minimized.

Various attempts to isolate mutants or variants resistant or tolerant to 2,4-D, for example, have met with limited success. Breeding herbicide-tolerant crop cultivars by conventional methods has not yet led to the production of major crops resistant to 2,4-D. Similarly, successful breeding for benzoic herbicides and MCPA resistance has not been reported (9). A few herbicide-tolerant variants have been selected in plant tissue culture (for review, see 15), but in only one case [carrot (*Daucus carota* L.) cells] did the variants stably retain the ability to tolerate 2,4-D. No resistance or tolerance to MCPA or dicamba was reported.

This absence of success is certainly due in part to our poor understanding of the molecular mechanisms involved in the metabolism (detoxification) of phenoxy acid and benzoic acid compounds in plants. It is generally assumed that benzoics, such as dicamba, are first hydroxylated and subsequently conjugated with glucose. Decarboxylation has also been reported (1). Interestingly, 5-OH dicamba was found to be nontoxic (1). In the case of 2,4-D ring hydroxylation, side chain cleavage as well as conjugation with glucose or amino acids all seem to participate in the detoxification process, whereas ring cleavage, which seems to occur in bacteria, has not been described in plants (1). Needless to say, genes coding for the above enzymatic functions has not been identified and characterized.

Another approach to the production of 2,4-D-resistant dicotyledonous plants would be to identify, clone, and transform into plant cells a bacterial gene known to code for the metabolism of 2,4-D into a less toxic compound. Genes coding for the degradation of Cba (chlorobenzoic acid), 2,4-D, and MCPA has been described in the bacteria *A. eutrophus* and was shown to be carried by a 77 kbp broad host range plasmid, pJP4, of the IncPl incompatibility group² (6, 10, 16). In addition, key functions involved in the degradative pathway have been identified by transposon insertion mutagenesis (7). Indeed, insertion of Tn1771 at several locations in two adjacent Hind III fragments of pJP4 inhibits either Cba degradation alone, or both Cba and 2,4-D degradation (5). This observation shows that 2,4-D and Cba are degraded via convergent pathways. Fragments of pJP4 known to be involved in 2,4-D degradation were cloned in pBR322 (recombinant designated JMP470)² and in pKT231 (recombinant designated pRD25)³ and were made available to us by these authors.

Plasmid-coded bacterial degradation of 2,4-D and presumably MCPA seems to occur via cleavage of the side chain followed by ring hydroxylation and ring cleavage (10). Presumably, Cba could also be hydroxylated and subsequently cleaved. Finally, the aromatic ring is converted into succinic acid. *A. eutrophus* harboring pJP4 can use Cba, 2,4-D, or MCPA as sole carbon sources. Once a key plasmid-borne gene is identified, it will be possible to transfer it to dicotyledonous plants.

This paper reports our preliminary results on the biological effects of a major 2,4-D metabolite, 2,4-DCP (2,4-dichlorophenol), as well as the strategy used to isolate the first *Alcaligenes* gene involved in the 2,4-D degradative pathway. Finally, we will briefly describe and exemplify the strategy used to transfer a bacterial gene into a legume, cowpea [*Vigna sinensis* (L.) Walp. 'Black Eye'].

MATERIALS AND METHODS

A. eutrophus (pJP4) and *E. coli* (pJMP470) were obtained from J. M. Pemberton. *E. coli* (pRD25) was obtained from R. H. Don. The latter plasmids were subcloned into pUC9

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² J. M. Pemberton. Personal communication.

³ R. H. Don. Personal communication.

or pUC19 and transformed into *E. coli* JM83 using the Xgal selection system (17). Metabolization of 2,4-D by *A. eutrophus* was analyzed by HPLC using the conditions derived from Drinkwine et al. (8) and by gas chromatography and electron capture detection. Cowpea leaf fragments were transformed in vitro by inoculation with *Agrobacterium tumefaciens* C59 carrying the disarmed pGV 3850 Ti plasmid (4). An *A. tumefaciens* C58 strain with a Ti plasmid harboring the dominant selectable marker *neo* gene (kanamycin resistance) was constructed by first cloning the Eco RI cassette from pMON213 containing the *neo* gene under the control of the *nos* T-DNA promoter (11) into the single Eco RI site of the intermediate vector pLGV2385 (18). The resulting recombinant DNA was introduced into *A. tumefaciens* (pGV3850) by a triparental cross. Exconjugants carrying a recombinant Ti plasmid pGV3850/*nos-neo* were selected and one of them, *A. tumefaciens* pGV3850 K2, was used to transfer the kanamycin resistance gene to cowpea cells. Selection of transformed plant cells was performed on Murashige and Skoog medium containing 1 mg/L 2,4-D, 100 µg/ml kanamycin, and 0.2 mg/ml cefoxitin in order to kill residual *A. tumefaciens* cells (3).

HPLC conditions. The mobile phase used was HPLC grade methanol with 0.01 M $\text{KH}_2\text{PO}_4:\text{K}_2\text{HPO}_4$, pH 6.7, both filtered through a 0.45-µm Millipore filter. Analysis was performed with a programmed gradient, at ambient temperature, with a 0 to 10% methanol gradient over 7 min, hold 1 min, then a 10 to 40% methanol gradient over 15 min at a flow rate of 2 ml per min. Separations were performed with a Whatman Partisil PXS 10/25 00S reverse-phase column with a Rainin 5-µm reverse-phase guard column on a Beckman modular HPLC system with ultraviolet detection at 280 nm.

Gas chromatography conditions. Analysis was performed on a Perkin-Elmer Sigma 3B gas chromatograph equipped with a ^{63}Ni -electron capture detector. The column used was SPB-5 nonpolar fused silica column 0.25-µm filter, 0.25-mm internal diameter, 30 m long, with a 1-m deactivated 0.25-mm internal diameter fused silica guard column joined with a capillary butt end connector. The carrier gas was 95% argon:5% methane or helium with 95% argon:5% methane makeup gas. Samples were analyzed on a linear temperature gradient; 95 C for 4 min, 4 C per min to 250 C. Injector port temperature was 250 C and detector temperature was 350 C.

Sample analysis. HPLC analysis was performed on 1-ml overnight cultures of *E. coli* JM83 containing pJMP470 in a succinate-Luria broth liquid medium containing 100 µg/ml 2,4-D free acid. The culture was pelleted at $1000 \times g$ for 5 min and washed two times with 5 ml distilled water. Washed pellets were resuspended in 1 ml distilled water. Ten milliliter cultures of *A. eutrophus* 134 containing pJP4 were grown in 2,4-D medium² at 29 C for approximately 48 h. The *A. eutrophus* cultures were pelleted at $1000 \times g$ for 5 min and resuspended in 1 ml distilled water. Cultures were extracted as described in Guerrant et al. (12). Samples were filtered with a 0.45-µm Millipore filter before analysis.

Gas chromatographic analysis was performed on 40 ml overnight culture of *A. eutrophus* 134 harboring pJP4 at 29 C in rich media (yeast extract and bactopectone) with 300 µg/ml 2,4-D or 2,4-D media. Cells were removed by centrifugation at $20\,000 \times g$ and the supernatant pH adjusted to 9 with NaOH. Samples were then extracted with two 20-ml volumes of anhydrous ether. The ether phase was dried with nitrogen gas. Dried samples were then derivatized with 100 µl bis-trimethyl silyltrifluoroacetamide and 100 µl glass-distilled pyridine in a 5-ml reaction vessel at 68 C for 20 min. Sample volume was then brought up to 5 ml with hexane and analyzed by gas chromatography.

RESULTS

Metabolization of 2,4-D by *A. eutrophus*. It has been proposed that the degradation of 2,4-D in soil bacteria proceeds first via the conversion of 2,4-D into 2,4-DCP by side chain cleavage (10). Figure 1 shows that indeed 2,4-DCP is a major metabolite produced by *A. eutrophus* JMP134 (pJP4) grown in minimal medium containing 2,4-D as sole carbon source. In fact, 2,4-DCP is excreted in large amounts into the culture medium whether the cells are grown in minimal or rich medium in the presence of 2,4-D (Figures 2 and 3). We have also observed that at a concentration of 0.3 mg/ml 2,4-DCP completely prevents growth of *A. eutrophus* JMP134 (pJP4)

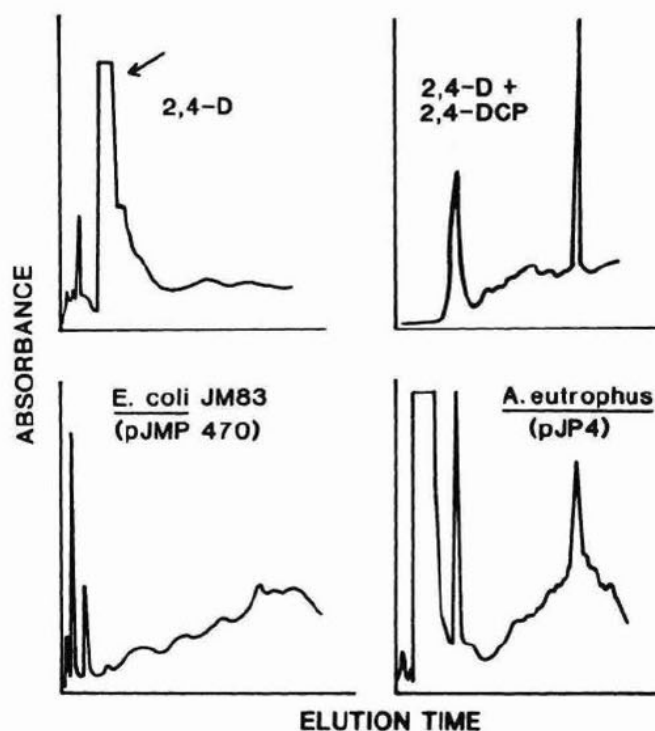


Figure 1. HPLC analysis of 2,4-D and 2,4-DCP in *E. coli* and *A. eutrophus*. Top left, 2,4-D standard; top right, 2,4-D plus 2,4-DCP standards. Bottom left, *E. coli* JM83 (pJMP470) grown in the presence of 2,4-D; bottom right, *A. eutrophus* (pJP4) grown in the presence of 2,4-D.

in rich medium. This observation strongly suggests that *A. eutrophus* possesses a system that precisely regulates the rate of uptake and degradation of 2,4-D as well as 2,4-DCP excretion. However, 2,4-DCP at 0.1 mg/ml was nontoxic. Figure 1 also shows that *E. coli* harboring pJMP470 (pBR322 containing some of the cloned 2,4-D degradation genes) does not take up 2,4-D. Similarly, *E. coli* 397 carrying pJP4 is unable to take up 2,4-D. This observation explains the failure of this *E. coli* strain to grow on 2,4-D medium (10).

Liu and Chapman (14) have reported that 2,4-DCP conversion into 2,4-dichlorocatechol is also plasmid encoded in *A. eutrophus*. This result, together with the above observa-

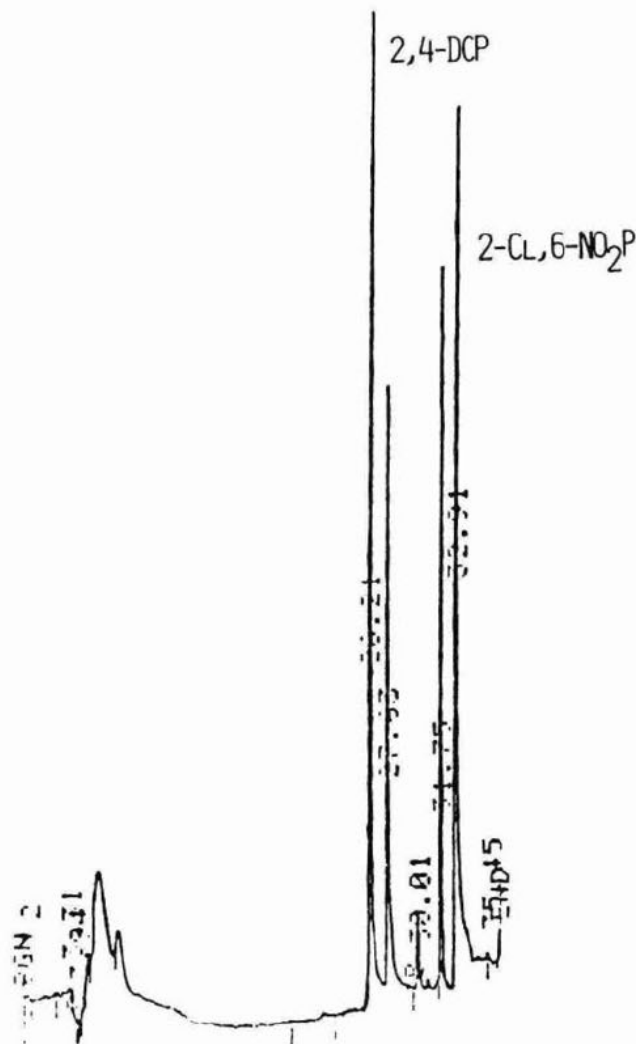


Figure 2. Gas chromatographic analysis of *A. eutrophus* minimal 2,4-D medium after bacterial growth. Labeled peaks are 2,4-DCP and the 2-chloro-6-nitrophenol internal standard. Carrier gas: 95% argon, 5% methane at 10 cm/min linear velocity; chart speed, 2 cm/min. Temperature gradient, 4 min at 95 C, 4 C/min to 200 C. Column, Supelco fused silica SPB-5, 0.25-mm internal diameter, 30 m with a 1-m guard column. Injector, 250 C; detector, 350 C.

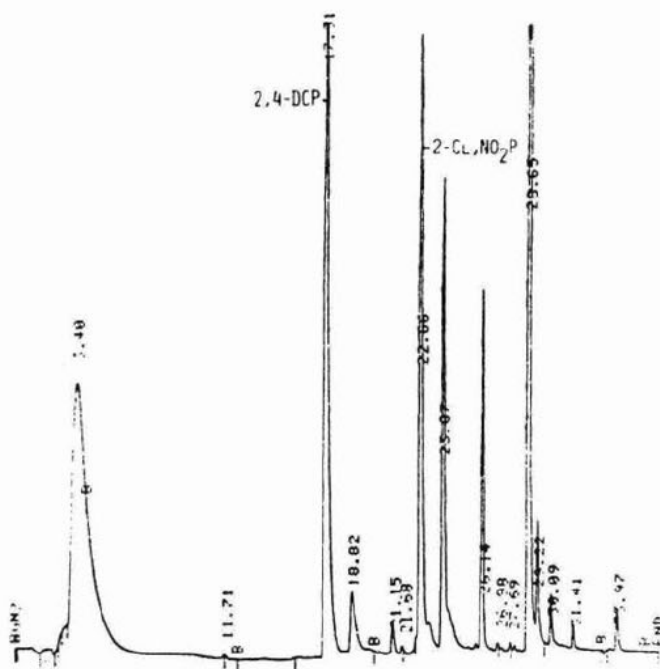


Figure 3. Gas chromatographic analysis of *A. eutrophus* rich medium with 2,4-D after bacterial growth. Labeled peaks are as in Figure 2. Carrier gas, helium at 22 cm/min linear velocity; chart speed, 5 cm/min. Other conditions as in Figure 2.

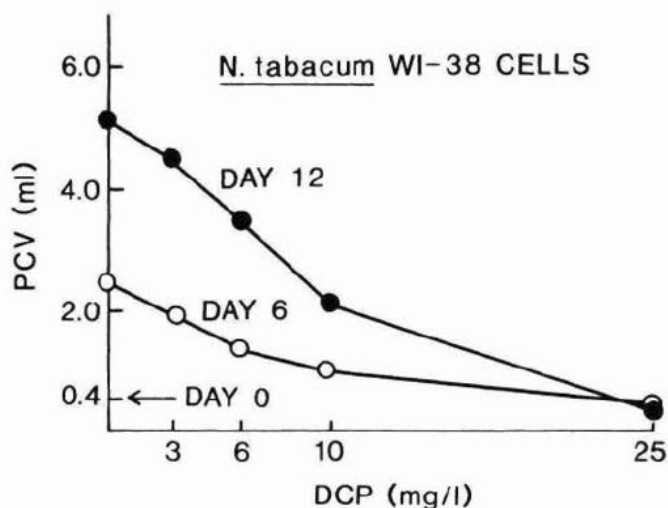


Figure 4. Growth of tobacco WI-38 cells in suspension in the presence of increasing amounts of 2,4-DCP. Arrow indicates inoculum size. Growth of cultures supplemented with 0 to 25 mg/l 2,4-DCP was followed for 16 days with samples removed for PCV measurement at 3-day intervals. For clarity, only PCV values for 0, 6, and 12 days are shown. The errors on PCV values are ± 0.1 ml for measurements up to 1 ml and ± 0.25 ml above ml.

tion, strongly suggests that, indeed, the first gene in the 2,4-D degradation pathway codes for an enzyme (a monooxygenase) which converts 2,4-D into 2,4-DCP.

Biological effects of 2,4-DCP on plants and plant cells. Assuming that the 2,4-D monooxygenase gene can be cloned from pJP4 and transferred into plant cells, it is expected that these plant cells will produce significant amounts of 2,4-DCP. Therefore, it is of paramount importance to de-

termine 2,4-DCP toxicity levels in plant cells. Growth of tobacco (*Nicotiana tabacum* L.) WI-38 cells in suspension culture was inhibited by increasing concentration of 2,4-DCP, 50% inhibition being observed at 10 mg/L 2,4-DCP (Figure 4). We also noted that 2,4-DCP did not exercise any hormonal action at lower concentrations.

The effect of 2,4-DCP on germinating WI-38 tobacco seeds was also investigated. We observed that 2,4-DCP had

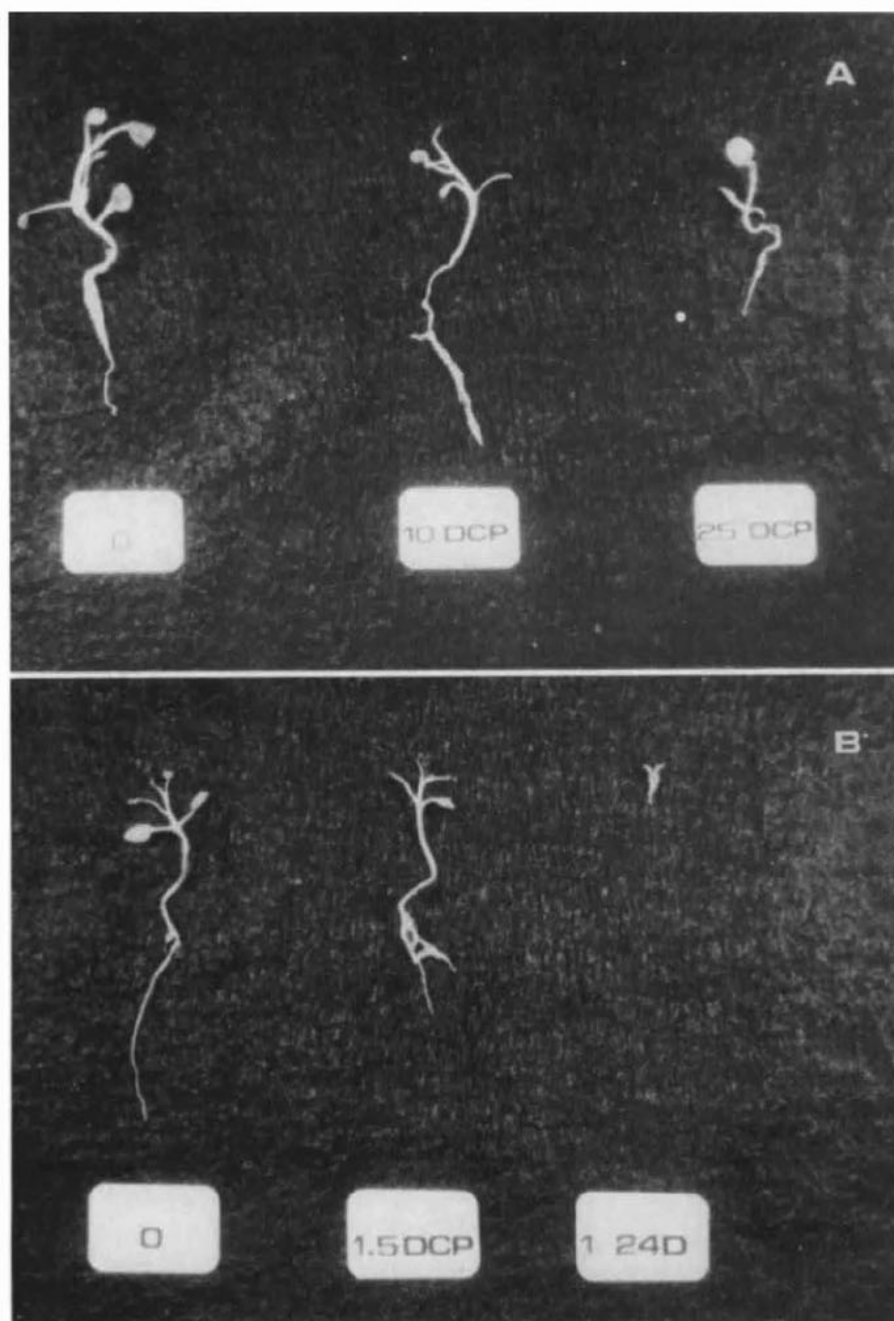


Figure 5. Effect of 2,4-D and 2,4-DCP on germinating tobacco WI-38 seeds. A. 0, control; 10, 10 mg/L 2,4-DCP; 25, 25 mg/L 2,4-DCP. B. 0, control; 1.5, 1.5 mg/L 2,4-DCP; 1, 1.0 mg/L 2,4-D.

Table 1. Tobacco WI-38 radicle and leaf length 10 days after germination as affected by either 2,4-D or 2,4-DCP^a.

2,4-D			2,4-DCP		
Concen- tration	Radicle	Leaf	Concen- tration	Radicle	Leaf
(mg/L)	(mm)		(mg/L)	(mm)	
0	24.3 ± 3.2	6.2 ± 0.6	0	23.1 ± 3.0	6.1 ± 0.6
1.0	2.1 ± 0.2	2.5 ± 0.2	1.5	24.1 ± 3.2	5.5 ± 0.5
2.0	... ^b	1.3 ± 0.1	3.0	19.3 ± 2.5	4.5 ± 0.5
4.0	... ^c	1.0 ± 0.1	10.0	19.5 ± 2.5	5.1 ± 0.5
10.0 ^d	...	...	50.0 ^d	...	...

^aData are means ± standard deviations.

^bRounded radicle; no measurements were made.

^cCallus at radicle.

^dNo germination at this concentration.

a much lower toxicity than 2,4-D as measured by leaf and root development as a function of 2,4-D and 2,4-DCP concentration (Figure 5). Table 1 shows that a pronounced toxic effect of 2,4-DCP is exhibited at a concentration of 10 mg/L. A 2,4-D concentration of 1.0 mg/L virtually blocks plant development. The same results were obtained with turnip (*Brassica napus* L.) seeds (not shown). In fact, we also observed that the toxic effect of 2,4-DCP is a transient one. It appeared that 3 weeks after germination, the inhibited plantlets resumed normal growth rate. Whether this is due to detoxification of 2,4-DCP or degradation in the medium is unknown.



Figure 6. Effect of painting 2,4-DCP on cowpea primary leaves. Left, control plant; right, painted plant, 10 mg/L 2,4-DCP.

The postgermination effect of 2,4-DCP application was tested in cowpea plants. For this, cowpea primary leaves were painted twice at a 13-day interval with a solution of aqueous 2,4-DCP (10 mg/L). Application of 2,4-DCP retarded the development of trifoliate leaves compared with control (Figure 6). Here also, this effect was transient.

Germinating wheat seeds are more sensitive to 2,4-D than to 2,4-DCP (Figure 7). However, the absence of a transformation system in economically important monocots,

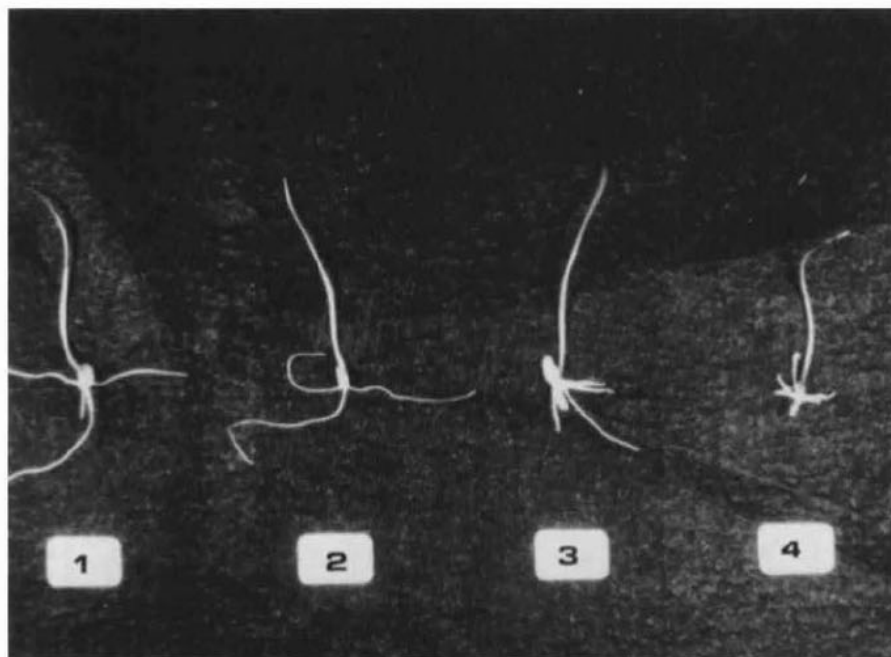


Figure 7. Effect of 2,4-DCP and 2,4-D on germinating wheat seeds: 1, control; 2, 1.5 mg/L 2,4-DCP; 3, 1.0 mg/L 2,4-D; 4, 1.5 mg/L 2,4-DCP + 1.0 mg/L 2,4-D.

with the possible exception of asparagus (*Asparagus officinalis* L.) (13), presently precludes the transfer of the 2,4-D monooxygenase gene into these plants.

Isolation of the 2,4-D monooxygenase gene. Don et al. (5) have demonstrated that the 2,4-D degradative pathway is clustered in a defined region of the *A. eutrophus* plasmid pJP4. This region has been cloned in various vectors and we have established an extensive restriction endonuclease map of these clones. In addition, we have subcloned the 2,4-D region (Figure 8). These subclones (designated pEPs) have been introduced into plasmidless *A. eutrophus* 228 rif via recloning into the wide host range mobilizable plasmid pUCD2. *A. eutrophus* exconjugants which became able to convert 2,4-D into 2,4-DCP have been identified by gas chromatography as described above. These experiments have shown that the 2,4-D monooxygenase gene is located on the subclones designated pEP109 through 111. The coding region of the 2,4-D monooxygenase gene will be identified by sequencing pEP111, and this gene will then be cloned next to a promoter recognizable by plant RNA polymerase II and transformed into plant cells as explained in the next section.

Transfer of a 2,4-D catabolic gene into plant cells. Recent progress in the knowledge of *A. tumefaciens*-mediated tumorigenesis has allowed the construction of disarmed Ti plasmids from which all oncogenes have been deleted and substituted by pBR322. Such Ti plasmids can be used to introduce foreign genes into the truncated T-DNA via homologous recombination with pBR322. It has been shown that bacterial genes under the control of an adequate promoter such as *nos* can be transferred to plant cells via T-DNA integration and are expressed in plants regenerated from these transformed cells (4).

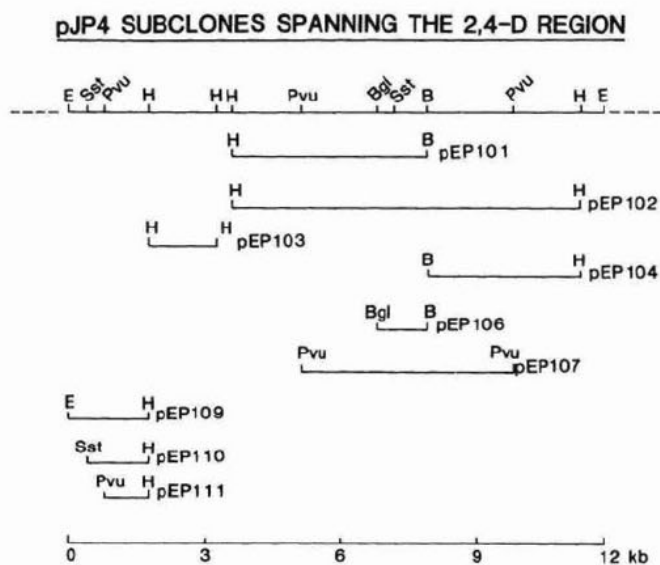


Figure 8. Map of the pJP4 2,4-D degradation region and subcloning scheme of the 2,4-D region. Top line: Eco RI fragment of pJP4 carrying the 2,4-D degradation genes. Restriction sites are as follows: E, EcoRI; Sst I, Sst I; Pvu, Pvu II; H, Hind III; Bgl, Bgl II; B, Bam HI.

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GENERAL STRATEGY FOR GENE TRANSFER

1. GENE A ISOLATED FROM pEP111
2. CLONE DOWNSTREAM FROM *nos* PROMOTER IN pLGV2385
3. CONJUGATE INTO *A. TUMEFACIENS* C58(pGV3850)
4. COCULTIVATE WITH LEAF DISKS
5. TEST TISSUE FOR 2,4-DCP PRODUCTION AND 2,4-D TOLERANCE

Figure 9. General strategy for the transfer of the 2,4-D monooxygenase gene (gene A) into plant cells.

The general cloning strategy is outlined in Figure 9. Briefly, a gene, preferably of known sequence, has to be cloned next to the T-DNA nopaline synthase promoter region in an intermediate vector such as pLGV2385. This molecule is then conjugated or transformed into *A. tumefaciens* pGV3850 whose T-DNA consists of the left and right borders flanking pBR322. Recombinants containing the foreign gene can then be used to infect plant cells which can in turn be used to assay for the presence and expression of this gene.

To exemplify this strategy, we have cloned an Eco RI cassette from pMON213 containing the *neo* gene under *nos* promoter control into the single Eco RI site of pLGV2385. The *nos-neo* hybrid gene has been used to confer kanamycin resistance to tobacco and petunia (*Petunia hybrida* Vilm.) cells (2, 11, 18).

After conjugation into *A. tumefaciens* pGV3850, recombinants containing this hybrid gene were selected and used to infect pieces of cowpea primary leaves in vitro. Callus cells actively proliferated on the leaf pieces infected with *A. tumefaciens* containing the *nos-neo* gene in the presence of 100 µg/ml kanamycin (Figure 10). Control leaf pieces incubated with *A. tumefaciens* pGV3850 not containing the *nos-neo* hybrid gene did not show callus cell proliferation, turned brown, and died on kanamycin-containing medium.

Once the *A. eutrophus* 2,4-D monooxygenase gene is identified and sequenced, a similar strategy will be used to transfer it into plant cells (Figure 9).

DISCUSSION

Catabolic plasmids have been shown to detoxify chemicals ranging from heavy metal salts to complex organic molecules. Thanks to the development of dicotyledonous plant transformation systems, it has become theoretically possible to transfer and express any prokaryotic gene in such plants.

Plasmid-borne genes coding for the degradation of chlorinated herbicides such as 2,4-D and MCPA have attracted attention since they offer the potential to produce herbicide-resistant plants. A prerequisite for the production of such plants is that the early degradation product(s) of 2,4-D be less toxic than 2,4-D itself. We have shown that 2,4-

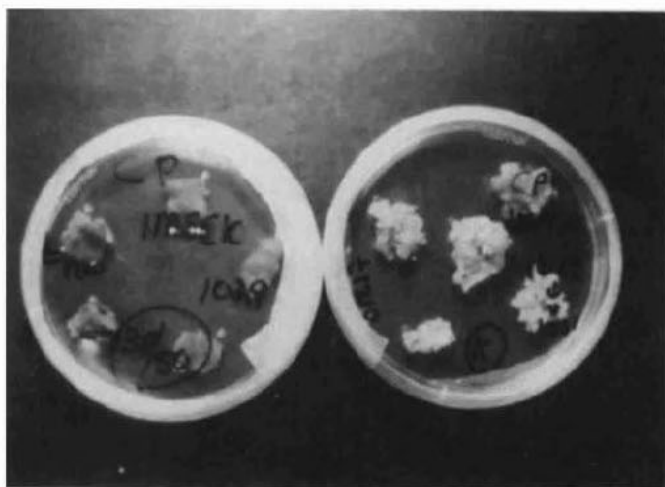


Figure 10. Result of a cocultivation experiment between cowpea primary leaf pieces and control *A. tumefaciens* pGV3850 (left) or *A. tumefaciens* pGV3850 containing a *mos-neo* hybrid gene in the T-DNA.

DCP, the first metabolite of 2,4-D in *A. eutrophus*, is indeed much less toxic than 2,4-D. Therefore, it appears justified to attempt the cloning and transfer of the 2,4-D monooxygenase gene into plants. In cases where it has been clearly demonstrated that the plant cells themselves can actively cleave the side chain of 2,4-D, it might be worthwhile to transfer the next two genes of the pathway which code for the two subunits of 2,4-DCP hydroxylase. These particular genes have been precisely mapped on the *A. eutrophus* plasmid pJP4 (5). In fact, one can even envision the transfer of all three genes into plant cells if it turned out that 2,4-dichlorocatechol was even less toxic than 2,4-DCP.

The above strategy represents an interesting alternative to the search for plant mutants resistant to chlorinated or other herbicides. Therefore, it would be of importance to search for plasmids able to degrade herbicides that do not belong to the phenoxy class.

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