A Simple and General Method for Transferring Genes into Plants

Abstract. *Transformed petunia, tobacco, and tomato plants have been produced by means of a novel leaf disk transformation-regeneration method. Surface-sterilized leaf disks were inoculated with an* Agrobacterium tumefaciens *strain containing a modified tumor-inducing plasmid (in which the phytohormone biosynthetic genes from transferred DNA had been deleted and replaced with a chimeric gene for kanamycin resistance) and cultured for 2 days. The leaf disks were then transferred to selective medium containing kanamycin. Shoot regeneration occurred within 2 to 4 weeks, and transformants were confirmed by their ability to form roots in medium containing kanamycin. This method for producing transformed plants combines gene transfer, plant regeneration, and effective selection for transformants into a single process and should be applicable to plant species that can be infected by* Agrobacterium *and regenerated from leaf explants.*

Efficient methods for introducing cloned genes into plants are important for understanding and controlling plant gene expression. The ability to manipulate genes could lead to rational, deliberate alterations of the genome of crop plants for improvement of their agronomic performance. Production of morphologically normal plants that contain and express foreign genes has been made possible by use of the natural gene-transfer capacity of *Agrobacterium tumefaciens*, a soil bacterium that causes crown gall disease in plants (*1*). Modified *A. tumefaciens* strains were used in which the tumor-inducing (Ti) genes had been deleted from the transferred DNA (T-DNA) and replaced with chimeric genes for bacterial antibiotic resistance that had been engineered to express in plant cells (*2*).

In previous studies the transformed plants were regenerated from calli derived from protoplasts (single cells without a cell wall) transformed by cocultivation with *A. tumefaciens* cells (*1*). However, the protoplast culture method has certain limitations: not all species of plants can be readily regenerated from protoplasts; the entire process can take up to 6 months from protoplast to plant; and plants derived from protoplasts can be subject to mutations or chromosomal abnormalities (*3*). Protoplast culture technology can also be difficult to reproduce in a new laboratory or to control from one experiment to the next. Transformation of stem or root explants in vitro is a simple substitute for cocultivation (*4*) but is laborious for large scale experiments and not easy to use with modified Ti plasmids that lack the tumor-inducing genes.

To overcome these limitations, we have developed an approach to transformation that integrates the gene-transfer capability of *A. tumefaciens* (*5*) with the simple and general regeneration capability of leaf explants (*6*). The construction of an *A. tumefaciens* strain (GV3Ti11SE) containing a modified octopine Ti plasmid (pTiB6S3SE) in which all phytohormone biosynthetic genes and the T_L-DNA right border sequence have been deleted has been described (*2*). Formation of a cointegrate between pTiB6S3SE and the intermediate vectors pMON120 or pMON200 results in a functional, avirulent T-DNA (*2, 7*). Plasmid pMON200 is a derivative of pMON120, which contains a translationally-improved chimeric NOS/NPTII/NOS gene for kanamycin resistance and confers a high degree of resistance to aminoglycoside antibiotics on transformed plant cells (*8*). The vectors also contain the nopaline synthase gene, which provides a second marker in the transformed plant cells (*1*).

Disks were punched from surface-sterilized leaves with a paper punch (6 mm in diameter) and submerged in a culture of *A. tumefaciens* grown overnight in luria broth at 28°C. After gentle shaking to ensure that all edges were infected, the disks were blotted dry and incubated upside-down on nurse culture plates prepared as described (*7*) containing medium that induces regeneration of shoots of the species being transformed. The age and titer of the bacterial inoculum had little influence on the effectiveness of the transformation; however, it was important to avoid excessive soaking of the internal tissues of the leaf disk by the bacterial culture. After 2 to 3 days, the disks were transferred to petri plates containing the same medium but without feeder cells or filter papers and containing carbenicillin (500 μg/ml) and kanamycin (300 μg/ml).

After 2 to 4 weeks, shoots that developed were excised from calli and transplanted to appropriate root-inducing medium containing carbenicillin (500 μg/ml) and kanamycin (100 μg/ml). Rooted plantlets were transplanted to soil as soon as possible after roots appeared. *Nicotiana tabacum* varieties Samson and Havana 425 (*9*) and a first-generation cross-fertilized (F_1) hybrid of *Petunia hybrida* (*10*) were easily transformed by this system. L2 tomato plants (*11*) responded better when the feeder plate medium was modified by reducing the amount of inorganic salts to one-tenth the usual concentration.

Uninoculated petunia leaf disks and those inoculated with *A. tumefaciens* strains containing pTiB6S3SE::pMON120 (which lacks the chimeric gene for kanamycin resistance) did not produce calli or shoots on medium containing 300 μg of kanamycin per milliliter (Fig. 1). In contrast, leaf disks inoculated with *A.*

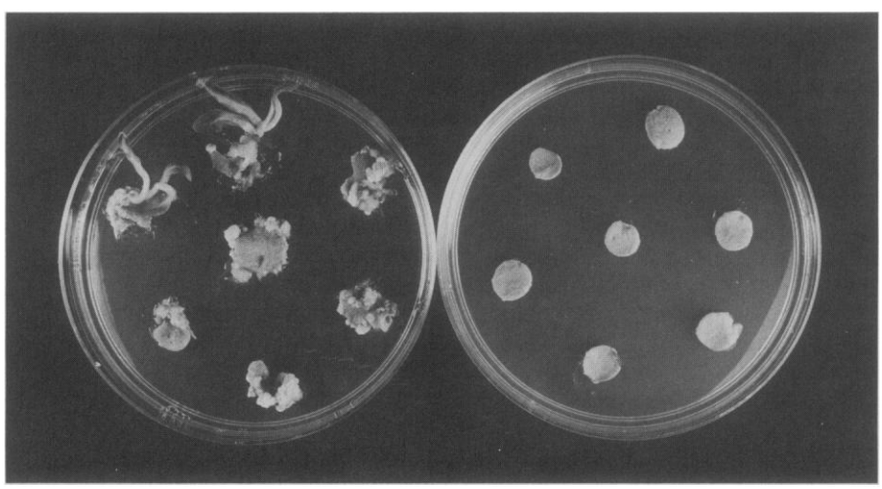

Fig. 1. Leaf disk transformation and selection of antibiotic-resistant cells. Leaf disks were punched from a surface-sterilized leaf of *Petunia hybrida* (Mitchell), inoculated with *Agrobacterium tumefaciens* strains, cultured on feeder plates, and transferred 2 days later to medium containing 300 μg of kanamycin per milliliter. The cultures were photographed 21 days after inoculation. Leaf disks transformed with pTiB6S3SE::pMON200 (which contains the chimeric gene for kanamycin resistance) are shown on the left, and disks transformed with pTiB6S-3SE::pMON120 (which lacks the kanamycin-resistance gene) are shown on the right.

tumefaciens strains containing pTiB6S-3SE::pMON200 rapidly produced calli and shoots on most of the wounded surfaces on the kanamycin-containing medium. All leaf disks were able to produce calli and shoots on control medium lacking kanamycin. Nopaline was detectable in all kanamycin-resistant calli.

In a typical experiment with *P. hybrida*, 25 of 69 independent shoots that grew from disks infected with pTiB6S-3SE::pMON200 rooted in the presence of kanamycin within 12 days. Of these 25 plants (see Table 1), 12 produced nopaline while grown in soil and 20 showed kanamycin resistance in the leaf callus assay, although 5 of these plants had delayed growth on kanamycin. Of the eight nopaline-negative, kanamycin-resistant plants, five showed induction of nopaline synthesis upon transfer of leaves to culture medium. Despite the frequent occurrence of these different phenotypes, it was easy to identify the desired transformants for studying gene expression, protein function, and inheritance of the inserted foreign DNA. For example, in this experiment, 12 of the 25 plants showed the expected pattern of constitutive gene expression. Similar results were obtained with tobacco.

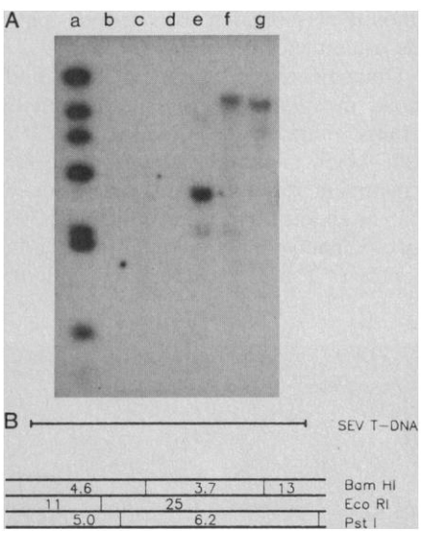

Fig. 2. (A) DNA blot hybridization analysis of tomato plants. Total plant DNA was extracted, purified by cesium chloride gradient centrifugation, and digested with restriction enzymes as described (*7*). After transfer of the DNA to nitrocellulose, nick-translated pMON200 DNA was used as a probe to identify the T-DNA fragments in the plant DNA. (Lane a) λ phage DNA digested with Hind III for size standards: (lanes b, c, and d) DNA from wild-type control L2 tomato plant digested with Bam HI, Eco RI, or Pst I, respectively; (lanes e, f, and g) DNA from an L2 plant (485) transformed with pTiB6S3SE::pMON200 as described (see text) and digested with Bam HI, Eco RI, or Pst I, respectively. (B) Restriction map of the pTiB6S3SE::pMON200 T-DNA region (SEV, split-end vector).

During regeneration from tissue culture, a meristem is thought to originate from a single cell and subsequent shoot regeneration to represent a clonal process in some but not all cases (*12*). When mesophyll protoplasts from three transformed plants were tested for kanamycin resistance, all of 100 colonies were resistant. This shows that the mesophyll cells were transformed but does not eliminate the possibility that other cells in the plant were not transformed. The question of meristem formation remains unresolved but is not a serious problem because the germ-line cells of all plants tested (petunia, tobacco, and tomato) were transformed, giving rise to transformed progeny in simple Mendelian fashion.

Progeny of transformed petunia plants inherit the inserted DNA and reflect the particular phenotype of their parent. One plant (2782) that showed constant expression of the gene for nopaline synthase and the chimeric gene for kanamycin resistance produced progeny that showed constant expression of both genes: 73 of 98 first-generation self-fertilized (S_1) progeny (74 percent) inherited the inserted DNA. In a backcross with the female parent as the transformant, 111 of 263 BC_1 progeny (42 percent) inherited the genes. Another petunia plant (2732) that showed culture-induced nopaline synthesis produced progeny with the same characteristic. In a sample of 24 S_1 seedlings germinated on water agar with MS salts, only 2 showed faint traces of nopaline. These same seedlings were transferred to callus-induction medium and assayed again 2 weeks later; 17 then showed strong nopaline production.

As with petunia and tobacco, some of the kanamycin-resistant tomato plants did not produce nopaline. The first transformed tomato plant grown to maturity in soil contained nopaline in all tissues (roots, stems, leaves, and flowers); leaf explants were resistant to kanamycin, and neomycin phosphotransferase activity was detected in crude extracts of leaves from this plant. Southern blot hybridization revealed the expected pMON200 DNA sequences in this plant (Fig. 2). Of 55 S_1 progeny, 42 produced nopaline. The leaf disk transformation method has been used successfully with several commercial tomato cultivars (genotypes) to produce transformed plants (*13*).

For many species, leaves provide a source of genetically uniform cells that have the capacity to regenerate whole plants when simple manipulations of the tissue culture are performed. The wounded edge of a leaf disk is susceptible to infection with *Agrobacterium* and, on an appropriate culture medium, is also a site of rapid cell division and induction of shoot regeneration. This results in effective targeting of transformation and regeneration to the same set of cells at the edge of the disk. The phenotype with kanamycin resistance provides an effective means to select for regeneration of transformed shoots or growth of transformed calli. By means of the leaf disk transformation system, transformed shoots could be obtained in 2 to 4 weeks and rooted plants in 4 to 7 weeks after leaf inoculation.

The high incidence of escapes was unexpected because, in controls, formation of calli and production of shoots were always completely inhibited on medium containing 300 μg of kanamycin per milliliter. The kanamycin-sensitive, nopaline-negative plants might have resulted from loss of DNA or loss of gene expression during development or from

Table 1. Transformed plants regenerated from petunia leaf disks infected with *Agrobacterium tumefaciens* strain containing pTiB6S-3SE::pMON200. These plants were produced as described (see text), grown to maturity in soil, and assayed for nopaline content and response to kanamycin (R, resistant; S, sensitive) by the leaf callus assay (*1*). Calli from leaf explants grown on medium without kanamycin were assayed to detect culture-induced production of nopaline.

Plant	Presence of nopaline		Response to kanamycin
	In soil-grown plants	In calli	
2817	–	+	R (delayed)
2818	+	+	R
2819	–	–	R
2820	+	+	R
2821	+	+	R
2822	+	+	R
2823	+	+	R
2824	–	–	S
2825	+	+	R
2826	–	+	R (delayed)
2827	+	+	R
2828	+	+	R
2829	–	+	R (delayed)
2830	+	+	R
2831	+	+	R
2832	–	–	S
2833	+	+	R
2842	–	–	S
2843	–	–	S
2844	–	+	R
2845	–	–	R
2846	–	+	R
2847	–	–	R (delayed)
2848	+	+	R (delayed)
2849	–	–	S

inefficient selection when transformed cells were near. Examination of four such plants revealed that three contained the expected DNA sequences homologous with pMON200, indicating that loss of foreign gene expression may account for many of the apparent escapes. The problem could be managed easily by first screening for plantlet rooting in the presence of 100 μg of kanamycin per milliliter and then applying the nopaline and leaf callus assays to assure stable expression in plants for further analysis and testing of progeny.

Unexpected changes in expression of T-DNA genes in plant cells and frequent loss of expression of opine synthase genes have been reported (*14*). Induction of genes for opine synthase upon transfer of explants to culture medium has also been observed (*15*). The predictable induction of these genes in certain of our transformants and the transmission of this property to progeny may be due to influences from surrounding DNA or chromatin structure at the site of insertion of the foreign genes into a plant chromosome. Such position effects have been observed frequently in other eukaryotic systems, such as transgenic mice (*16*) and *Drosophila* (*17*).

The leaf disk transformation system should be applicable to many of the plant species that are susceptible to *A. tumefaciens* and can be regenerated from leaf explants. By integrating the transformation, selection, and regeneration processes into a simple and efficient procedure, the production of transformed plants could become routine for studies of gene expression and of the physiology or biochemistry of plants, even in laboratories with little expertise in tissue culture methods.

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9. Leaf disks were incubated on nurse culture plates with medium (*p*H adjusted to 5.7) containing MS salts (Gibco), B5 vitamins, sucrose (30 g/liter), benzyladenine (1.0 μg/ml), naphthalene acetic acid (0.1 μg/ml), and 0.8 percent agar. Shoots were rooted on similar medium lacking phytohormones.
10. The F_1 hybrid petunia was produced by crossing lines V23 and R51. The same media were used for petunia as for tobacco (*9*).
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18. We thank the Department of Genetics, University of Amsterdam for the F_1 hybrid petunia and D. Pratt for the L2 tomato plants.

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Phagocytes as Carcinogens: Malignant Transformation Produced by Human Neutrophils

Abstract. *In a study of the relation between chronic inflammation and carcinogenesis, C3H mouse fibroblasts of the 10T 1/2 clone 8 line (10T 1/2 cells) were exposed to human neutrophils stimulated to synthesize reactive oxygen intermediates or to a cell-free enzymatic system generating superoxide (xanthine oxidase plus hypoxanthine). After exposure, the 10T 1/2 cells were either placed in tissue culture or immediately injected into athymic nude mice. Both malignant and benign tumors developed in the mice injected with treated cells, but not in those injected with control cells; in one instance cells grown from one of the benign tumors subsequently developed a malignant phenotype. Malignant transformation was also observed in treated cells in the experiments in vitro.*

We showed previously that human phagocytes can produce mutations in bacteria and mutations and sister chromatid exchanges in cultured hamster ovary cells, and that reactive oxygen metabolites were important for the production of these genetic lesions (*1–4*). The results suggested that the toxic intermediates or by-products of oxygen metabolism generated by inflammatory phagocytes may play a role in the carcinogenic process. The findings of other groups concur with these observations (*5–10*). We conducted the present study to address this question more directly.

The cells used were C3H mouse fibroblasts of the 10T 1/2 clone 8 line (10T 1/2 cells). At passages 10 through 12, 10T 1/2 cells were grown in monolayer culture and treated as shown in Table 1. After the treatment the cells were washed, removed from the petri dishes, and plated according to standard methods for transformation assays (*11–13*). After 6 to

Table 1. Transformation of C3H 10T 1/2 cells in vitro. The C3H 10T 1/2 cell line and transformation assay methods were described previously (*11–13*). Briefly, plateau-phase ($\sim 4 \times 10^4$ cells per square centimeter) cells, grown in Eagle's basal medium containing 10 percent fetal calf serum, were treated while they were attached to plastic 100-mm petri dishes. Neutrophils were prepared from healthy human volunteers as described (*20*). In general, target 10T 1/2 cells were incubated for 60 minutes at 37°C with neutrophils and with or without TPA (Consolidated Midland; 1.0 μg/ml). In some experiments, xanthine oxidase (15 μg/ml) and hypoxanthine (7 μg/ml), both from Sigma, were layered onto the 10T 1/2 cells instead of neutrophils. At the end of the incubation period the 10T 1/2 monolayers were washed, removed from the dishes, and plated into 100-mm dishes at densities such that less than 2000 surviving cells per dish were expected (on the basis of plating efficiency determinations). Medium was changed twice weekly until confluence was reached, then weekly thereafter until fixation and staining were performed at 6 to 8 weeks.

Treatment	Number of cells surviving per dish	Total number of dishes	Dishes with type III foci	
			Total	Percentage
None (control)	1265	210	7	3.3
Hypoxanthine plus xanthine oxidase	1335	20	3*	15.0
Neutrophils (10^6)	1380	20	2	10.0
Neutrophils (5×10^6)	1200	20	10*	50.0
Neutrophils plus TPA	1320	20	7*	35.0
Neutrophils (5×10^6) plus TPA	480	20	3*	15.0
TPA (1 μg/ml)	1005	20	1	5.0

*Significantly greater ($P < .05$) transformation frequency than untreated control (*21*).