

## Leaf disc transformation of cultivated tomato (*L. esculentum*) using *Agrobacterium tumefaciens*

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### Abstract

The leaf disc transformation/regeneration system was modified for tomato (*L. esculentum*). Both leaf explants and cotyledon/hypocotyl sections can be used to regenerate transformed plants. We have obtained over 300 transgenic plants from eight tomato cultivars. We have evidence for both single and multi-copy insertions of the T-DNA, and have demonstrated inheritance of the T-DNA insert in the expected Mendelian ratios. Several heterologous promoters function in tomato. A reduced efficiency of transformation was observed with binary T-DNA vectors as compared to co-integrate T-DNA vectors. The ease of the leaf disc method makes tomato a premier experimental organism for plant biotechnology.

### Abbreviations

BA, benzyl adenine; IAA, indole acetic acid; LB, Luria Broth.

### Introduction

Tomato (*Lycopersicon esculentum*) is a valuable species for studying plant biology because it will permit the integration of the tools and concepts of genetics, plant physiology, developmental biology, host-pathogen interactions, molecular biology and genetic engineering for studying and manipulating all of these processes. Tomato is well characterized genetically (Rick, 1984) and isogenic stocks are available (Maxon Smith and Ritchie, 1983) for many traits of interest, such as mutants in fruit ripening, male sterility and disease resistances.

Tomato is susceptible to *Agrobacterium* infection, and is therefore amenable to current plant transformation techniques. Cultured leaf explants of cultivated tomato easily give rise to shoots (Behki and Lesley, 1976; Kurtz and Lineberger, 1983), allowing the application of the leaf disc transformation method (Horsch et al., 1985). This method has largely obviated the need to develop a protoplast to plant regeneration system for tomato transformation, although such procedures have recently been published (Shahin, 1985; Niedz et al., 1985).

We have modified the leaf disc system for tomato, and have obtained over 300 transformed tomato plants from eight commercial cultivars. The advantage of an easy transformation/regeneration system for tomato is that it will allow molecular biologists to characterize, in detail, the function and expression of important homologous and heterologous genes or promoters in a crop species.

### Materials and Methods

#### Plant Material

Tomato seeds were purchased from Hummert's Seed Co., St. Louis, or were obtained from John Hewitt, Vegetable Crops Dept., U. California-Davis. The lines used were Marglobe, Rutgers, F1 Hybrid Beefmaster VFN, F1 Hybrid Betterboy VFN, F1 Hybrid Burpee's Bigboy, F1 Hybrid Floramerica VF, F1 Hybrid Mocross Surprise, F1 Hybrid Perfect Peel, Roma Italian Canner, UC134-1-2, LA1563, VF145B-78-79, UC82b, UC204A, UC204B, and VF36. Seeds were germinated in soil, transplanted at the two-leaf stage to 4-in. pots, and then to 2-gallon pots. Plants were grown in greenhouses, and fertilized weekly with 1/10X Peter's (15-15-15).

### Shooting Response

The tomato lines were screened for their ability to regenerate shoots from leaf sections, using published media compositions (Kurtz and Lineberger, 1983; Evans and Sharp, 1983). The culture medium (MSO) contained MS salts (Gibco), B5 vitamins, 3% sucrose, 0.8% agar, and one of the following hormone compositions: A (5mg/l BA; 0.2 mg/l IAA), B (5mg/l BA; 1.0 mg/l IAA), C (2.5 mg/l BA; 0.2 mg/l IAA), D (2.5 mg/l BA; 1.0 mg/l IAA), E (1.0 mg/l BA; 0.2 mg/l IAA), or F (4 mg/l Kinectin; 4 mg/l IAA).

### Bacterial Strains

Disarmed *Agrobacterium tumefaciens* strains (Fraley et al., 1985) containing pMON200SE (Horsch et al., 1984) or derivatives (Table 1) were used for transformations. Binary vectors (pMON505 or derivatives) (Horsch et al., submitted) were also tested. Overnight cultures (LB containing 50 µg/ml spectinomycin and streptomycin) were used for transformations.

**Table 1:** *Agrobacterium* strains and plasmids used for tomato transformation.

<u>Agrobacterium</u>	<u>Host</u>	<u>Ti plasmid</u>	<u>Intermediate vector</u>	<u>Selectable marker promoter-gene-3'</u>
GV3111SE <sup>a</sup>	C58C1	pTi6S3SE	pMON200SE <sup>b,c</sup> pMON259SE pMON274SE pMON280SE	NOS-NPTII-NOS SoySS-NPTII-NOS PeaSS-NPTII-NOS PetSS-NPTII-NOS
A208 <sup>c</sup>	C58C1	pTiT37SE(ASE) <sup>f</sup>	pMON505 or derivs. <sup>d</sup> pMON200SE or derivs.	

<sup>a</sup> DeGreve et al, 1981

<sup>b</sup> Fraley et al, 1985

<sup>c</sup> This is the prototype plasmid, all others are derivatives at the NPTII site.

<sup>d</sup> Horsch et al., submitted. pMON505 is a binary vector, all others are co-integrate.

<sup>e</sup> Scialy et al., 1977

<sup>f</sup> Rogers et al., in preparation

### Co-cultivation of *A. tumefaciens* and Tomato Leaf Sections

The 2 sub-terminal leaflets from the 4th, 5th, and 6th nodes of 6-week old greenhouse grown plants were used for transformation. Leaves were allowed to air dry in a laminar flow hood for 30 minutes, sterilized for 10 minutes in 10% Chlorox in 0.1% Tween 20, and rinsed 3 times with sterile distilled water. The leaves were cut with a sterile scalpel into squares approximately 2 x 2 cm. This size of explant gave better results than leaf discs from a hole punch. Leaf pieces were pre-conditioned, abaxial side up, on feeder plates (100 x 15 mm) (Horsch et al., 1985) containing 1/10X the inorganic salts of MSO, B5 vitamins, 3% sucrose, 0.8% agar, and appropriate hormones. After 2 days pre-conditioning, the

leaf pieces were removed from the feeder plates and gently mixed with a diluted (1:20 or greater with MSO liquid media) overnight culture of *Agrobacterium*, until the leaf edges look slightly wet. The dilution is a fairly critical parameter for tomato transformation and helps prevent *Agrobacterium* from overgrowing the leaf sections during subsequent culture. The overnight cultures are standardly  $3-4 \times 10^9$ /ml, thus a 1:20 dilution gives a  $1-2 \times 10^8$ /ml concentration. Transformations have been effective at dilutions as low as  $10^6$ /ml. The leaf pieces were removed from the *Agrobacterium* cells and thoroughly blotted dry between sterile filter papers. The leaf pieces were then returned to the same feeder plates for a two-day co-cultivation period. After two days the leaf pieces were transferred to selection plates (full strength MSO, appropriate hormones, 500  $\mu$ g/ml carbenicillin and 100  $\mu$ g/ml kanamycin). Leaf pieces with developing callus were transferred every three weeks to selection plates (100 x 25 mm) until shoots developed. Shoots were excised from surrounding callus and cultured on rooting media (MSO containing 100  $\mu$ g/ml kanamycin). Shoots that rooted in the presence of kanamycin were transferred to soil for further analysis. In general, transformation and subsequent regeneration was more efficient with greenhouse plants grown in the spring and fall, as compared to winter and summer. Cotyledons and hypocotyl sections from aseptically-grown 10 day old seedlings have also been used successfully for transformations.

#### Expression Assays

The presence of nopaline in transformed tomato leaf tissues was assayed by paper electrophoresis (Ottens and Schilperoord, 1978). We assayed the plants about one month after transfer to soil, in order to insure that the plant was producing nopaline, rather than having transported nopaline from the callus. Kanamycin resistance was assayed by culturing sterile leaf explants of putative transformants on selection media (100  $\mu$ g/ml kanamycin and appropriate hormones). Leaves of transformed plants would callus within 1-2 weeks, whereas untransformed leaves form no callus and often turn yellow. Neomycin phosphotransferase II assays (Reiss et al., 1984) were also used to assess the expression of the chimeric NPTII gene.

#### DNA Analysis

DNA was isolated as in Maniatis et al. (1984), or as in Zimmer and Newton (1982). Southern hybridization analyses were performed as in Maniatis et al. (1984), on nitrocellulose paper, using the appropriate pMON vector DNA as a nick-translated probe.

#### Plant Terminology

All plants regenerated from a single leaf piece were considered to be from a single transformation event. From the first transformation experiments all plants developing from a leaf piece were saved, even though this could result in several different phenotypes (Table 4). Later experiments were restricted to one regenerated plant per leaf piece, to assure that each regenerate represented an independent event. Using the designations suggested by Chaleff (1981), the primary transformants are the R0 generation. Progeny of the R0 are the R1, and progeny of the R1 are the R2. The transformed plants were allowed to self-pollinate by open pollination, or they were backcrossed to emasculated untransformed plants.

## Result and Discussion

### Transformation and Regeneration

Before attempting transformation experiments, the tomato lines were screened for their ability to regenerate shoots from leaf sections. The shooting responses are summarized in Table 2. After initial tests of all six media in transformation experiments, two (D and E) were chosen for subsequent experiments, both for convenience and because they gave the best shooting responses. Medium D was used for tomato lines 1 through 9 (Table 2), and medium E for lines 11 through 16. VF36 was not included in the initial regeneration analyses, but performs well on both D and E media.

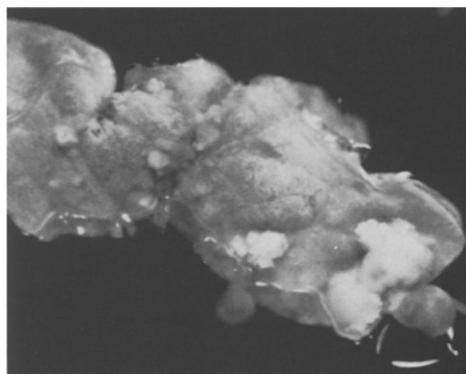
Transformation experiments could be scored as early as 3 weeks after selection. Callus could be seen (Figure 1) at the cut edges of the leaf and also at wound sites where tissue was damaged during the inoculation process. Often the leaf edges gave rise to green structures that directly developed into shoots. Upon further sub-culture the callus eventually developed into or produced compact green structures that formed shoots. On the average (all genotypes and transformations) it took about 3 months from transformation to establishment in soil. Normally about 10-20% of the initial leaf pieces inoculated gave rise to plants established in soil, although a much higher percentage of the leaf pieces formed callus. For example, in one experiment, 80 leaf pieces of VF 36

were inoculated with a pMON200SE derivative. After three weeks of selection, there were 76 independent calli on these 80 leaf pieces. After an additional 9 weeks of selection, there were 24 remaining calli, several of which were developing shoots.

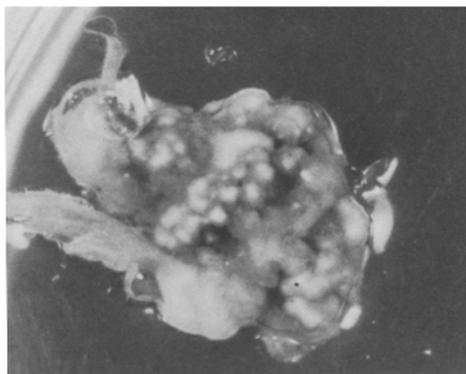
**Table 2:** Shooting response of tomato leaf pieces. Leaf pieces were scored after 3 weeks of culture on MSO medium and varying hormone concentrations. The number of leaf pieces forming shoots/total number of leaf pieces is indicated. The \* indicates the better media for each tomato line, as assessed by the number of shoots per leaf piece and the appearance of the shoots.

Tomato Line	Medium						
	(untransformed)	A	B	C	D	E	F
1. Marglobe		20/20*	11/15	20/20	15/20*	6/14	10/20
2. Rutgers		13/15	15/15	10/15*	12/14*	8/14	15/15
3. Beefmaster		23/24	22/25	20/25	24/25*	22/25*	12/24
4. Betterboy		20/25	15/20	12/20	17/25*	16/20*	14/25
5. Bigboy		13/20*	3/10	12/20	10/25	2/15	4/16
6. Floramerica		8/20	3/15	9/15*	13/20	5/15	2/15
7. Mocross		4/15	6/15	8/15	19/25*	3/15	4/15
8. PerfectPeel		15/15*	13/15	15/15	15/15*	9/10	14/25
9. Roma		12/15	4/15	20/24*	11/15	9/15	7/15
10. UC134-1-2		15/15	14/15	15/15*	5/5	10/10*	14/14*
11. LA1563		10/10	7/10	10/10*	9/10*	11/11*	11/12
12. VF145B-78-79		6/14	15/15	12/15	14/15	9/17*	13/15
13. UC82b		17/20	18/20	18/20*	19/20	20/24*	20/23
14. UC204A		5/5	3/5	5/5	5/5	6/6*	4/5
15. UC204B		7/15	6/20	8/20	12/20	11/22*	10/25

**Figure 1:** (a) Callus and "incipient shoot" forming on leaf piece (VF 36, pMON200SE derivative) 3 weeks after selection.



(b) Proliferating callus and shoot regeneration (Beefmaster, pMON200SE) 10 weeks after selection.



The majority of the putative transformed shoots were able to root in the presence of 100  $\mu$ g/ml kanamycin. Some calli died upon subsequent subculture, and some shoots did not root on kanamycin. These are probably the result of either unstable expression of the resistance gene, or to "escapes" from the initial selection. Callus often developed on

regions of the leaf that were not in direct contact with the medium, but died when subcultured and brought into more direct contact with the kanamycin. The longer culture period (as compared to *Petunia*) required for transformed shoot development in tomato has the advantage of longer selection and thus a reduction in the number of apparent "escapes".

The different genotypes were variable in their ability to form shoots from transformed leaf pieces and in the length of time required in culture before shoots could be established in soil. This ranged from as short as 6-10 weeks for Perfect Peel, VF36 and UC134-1-2, to as long as 6 months for Roma. Once a leaf piece started forming shoots it was easy to obtain multiple shoots. The variability in shoot development after transformation is in contrast to the shooting ability of the genotypes per se; all genotypes rapidly developed shoots that could be transferred to soil within two months in the initial hormone grid experiment (Table 2). The genotypes showing the best regeneration properties in transformation experiments were Beefmaster, Perfect Peel, UC134-1-2, UC82b, and VF36. Our experiments were soon limited to these genotypes because they yielded the most efficient results. However, we have also obtained transformed plants from Roma Italian Canner, Rutgers, and Betterboy. We expect that most commercial cultivars will be amenable to transformation, although modifications of hormone levels or other culture conditions might be required to optimize results.

We have also been able to transform cotyledon and hypocotyl explants with an efficiency comparable to that of leaf discs. For example, in one experiment with VF36 and a pMON200SE derivative, 48 of 132 cotyledon pieces, and 43 of 121 hypocotyl sections showed callus or green incipient shoots after 5 weeks of selection.

#### Cis versus Binary Vectors

Both the GV3111 (octopine strain) and A208 (nopaline strain) *Agrobacterium* strains are effective in tomato transformation. However, several experiments have indicated that the pMON505 or derivatives) binary vectors do not work as well in tomato as do the cointegrate (cis) vectors. A comparison using the cultivars Perfect Peel and VF36 indicated at least a five-fold reduction in callus formation during the initial three weeks of selection (Table 3). Several other experiments (data not shown) have shown even lower transformation frequencies with these binary vectors. Given that only 10-20% of initial calli go on to produce shoots, it is clear that these binary vectors will only be useful in tomato if large numbers of leaf pieces are inoculated (we have not yet obtained a transgenic plant from a binary vector transformation). These binary vectors result in satisfactory transformation frequencies in *Petunia* leaf disc transformation (Horsch et al, submitted). It is possible that the higher efficiency of *Petunia* transformation masks a difference between the cis and binary vectors. Differences in transformation efficiencies in tomato may be related to the stability and/or size of these binary vectors; these parameters are being tested.

**Table 3.** Comparison of cointegrate (cis) and binary (trans) vector transformation. Callus formation was scored after 3 weeks of selection.

Cultivar	Vector	#Leaf pieces with callus/ total leaf pieces
VF36	pMON200SE (cointegrate)	23/43 (53%)
	pMON505 (binary)	2/26 (7%)
	pMON200ASE (cointegrate)	13/32 (40%)
Perfect Peel	pMON200SE	10/17 (59%)
	pMON505	3/21 (14%)
	pMON200ASE	5/32 (16%)

#### Analysis of Transformed Plants

The vast majority (>90%) of the transformed plants generated from leaf transformation appeared normal phenotypically and produced fruit and viable seeds. A few primary transformants appeared somewhat abnormal (stunted growth or club-shaped leaves). These morphological abnormalities did not transmit genetically.

From 50 leaf pieces (events) a total of 171 plants were obtained. Representative events are shown in Table 4. Of these plants, 11 were apparent "escapes", 117 expressed both the nopaline and kanamycin resistance markers, 41 expressed only the kanamycin resistance marker and 2 plants expressed only the nopaline marker. Inasmuch as all plants were required to root in the presence of kanamycin before they were transferred to soil, it could be that the 13 kanamycin sensitive plants had

lost, or turned off the expression of the kanamycin resistance gene. Such phenomena have been observed previously in *petunia* (Horsch et al, 1985).

Several of the leaf pieces gave rise to multiple shoots. For example, in one case (event 12) we obtained 26 nopaline negative, kanamycin resistant plants, as well as one nopaline negative, kanamycin sensitive plant and two nopaline positive, kanamycin resistant plants. Because of these three different phenotypes, it is probable that that particular leaf piece actually gave rise to at least three independent transformation events. Four of the 26 nopaline negative, kanamycin resistant plants were examined on Southern (data not shown), and all four (Plants 13, 24, 44 and 50) showed identical hybridization patterns, as would be expected of plants from the same transformation event.

**Table 4:** Representative examples of the phenotypes, and numbers of transformed plants produced. Each leaf piece is designated as an event. Nop+ indicates nopaline positive and, Nop- indicates nopaline negative plants. KanR indicates kanamycin resistant, and KanS indicates kanamycin sensitive plants.

Event	Genotype	Nop+		Nop-	
		KanR	KanS	KanR	KanS
1	Beefmaster	4		1	
4					1
10				3	
12		2		26	1
15	Perfect Peel	2			
18		6			
20			1		
23		7			
24		5		1	1
29		10		1	1
30		7	1	1	
33		1		3	
37		8			
44		3			
45	VF36	3			

#### Expression

As in *petunia* (Horsch et al, 1985) we have observed plants that have either lost, or turned off nopaline expression. We have not been able to re-induce nopaline expression in these plants, either by 5-azacytidine treatment (Hepburn et al., 1983), or by inducing callus formation.

The three heterologous small subunit promoter fusions (pMON280SE, pMON259SE and pMON274SE) and the NOS promoter (pMON200SE) all confer kanamycin resistance in tomato, as assayed with the leaf callus assay. It is difficult to quantify promoter strength with the leaf callus assay. To compare the promoters more quantitatively we have used the NPTII gel overlay assay. Several factors other than absolute promoter strength can determine the level of gene expression, including copy number and position of insertion (Jones et al., 1985). In general we can state that the small subunit promoter fusion constructs show much higher levels of NPTII activity than do the pMON200SE constructs, when averaged over several plants. We have used the NPTII assay as a rapid method to identify plants with high levels of NPTII expression driven from the relatively weak NOS promoter; such plants have proven to be multi-copy insertions. This assay could also be used in this way to identify "good" chromosomal sites for expression from single-copy inserts.

#### Inheritance

Mendelian inheritance of foreign genes in *petunia* (Horsch et al., 1984) and tobacco (De Block, et al., 1984, Potrykus et al., 1985) has been demonstrated. Seed samples of R1 progeny, and R0 x untransformed backcross progeny from 18 independent transformants were assayed to determine the stability and inheritance of marker genes in tomato (Table 5). The inheritance data show that the T-DNA insert was always transmitted to the next generation and expressed, but not always in the ratio expected for a single dominant gene. Several plants showed skewing towards nopaline positive progeny, and suggested insertion at more than one chromosomal location (see below). In no case was the T-DNA lost in the R1 or R2 generations, and in no case was there a skewing towards nopaline negative progeny.

**Table 5:** Segregation ratios from seed samples of independent primary transformants. The \* indicates that the leaf assay for kanamycin resistance was used, because the plant did not express the NOS gene.

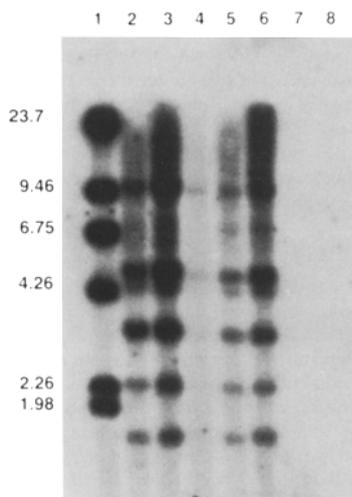
Event	Genotype	Plant #	Self-pollinated		Backcross	
			Nop+	: Nop-	Nop+	: Nop-
1	Beefmaster	3	40	: 10	59	: 50
2		5	57	: 14		
3		152	20	: 3		
10		225	21	: 5		
12	Betterboy	50 *	16	: 6		
17		197	38	: 14		
14		198	40	: 12		
15		215	25	: 8		
16		234	32	: 11		
18	Perfect Peel	196	48	: 13		
18		211	46	: 20		
23	Roma	206	41	: 15		
24	UC134-1-2	55	25	: 2		
27		63 *	50	: 19	61	: 58
25		107	40	: 16		
33		110 *	23	: 2		
36		117	20	: 10		
37		121	27	: 0		
39		221	28	: 3		
44	UC82b	135	80	: 25	80	: 89

Nopaline positive progeny of R1 plants exhibiting a 3:1 ratio were self-pollinated and assayed in order to identify homozygous plants. Of 4 R1 plants tested from plant 3, one gave a R2 ratio of 83 nopaline positive: 0 nopaline negative, indicating a probable homozygote. Homozygous plants will be used to assess gene stability in subsequent generations.

#### Southern Analysis

We have examined several of the R0 plants and their progeny to assess the copy number of the T-DNA inserts, and to correlate the structure of the T-DNA with the inheritance data. We have evidence of single copy insertions, multi-copy linked insertions, and multi-copy unlinked insertions. Southern analysis of plant 135 (event 44) (Figure 2) confirmed the presence of the T-DNA. The appearance of both the internal 3.7 kb BamHI fragment, and six border fragments suggest that there are three monomeric inserts of T-DNA. Dimeric inserts should show a diagnostic 6.3 kb fragment. Nontransformed UC134-1-2 and nopaline negative progeny of plant 135 showed no hybridization signals.

**Figure 2:** Southern analysis of plant 135 (UC82b, pMON243SE) and R1 progeny. DNA was digested with BamHI. Lane 1) HindIII digested lambda DNA, lane 2 Plant 135, lanes 3 - 6) nopaline positive progeny lane 7) nopaline negative progeny, lane 8) nontransformed UC82b. The weak signals in lane 4 are due to a low amount of DNA loaded on the gel.



Segregation of junction fragments in the nopaline positive progeny might be expected if plant 135 contained unlinked copies of the T-DNA. Because there was no segregation of junction fragments in 14 nopaline positive progeny, the T-DNA inserts were probably linked.

Plant 121 (event 37) gave an R1 ratio of 27:0, suggesting multiple inserts. Southern analysis of this plant (data not shown) showed the expected 3.7kb internal BamHI fragment, as well as multiple bands (>10) representing junction fragments. Six of the 27 R1 progeny of plant 121 were also examined. All 6 showed the 3.7kb internal BamHI fragment. Among these 6 progeny there were at least two different subsets of the parental junction fragments, implying that at least two functional copies of the T-DNA insert are undergoing independent assortment during meiosis.

Southern analysis of 6 other primary transformants (data not shown) indicate that both single (Plants #63, 107 and 102) and multiple (Plants #69, 110 and 152) insertions occurred. Independent transformants had distinctive patterns of junction fragments, as expected from random insertions of the T-DNA. While it is not obvious why certain transformants have multiple copies of the T-DNA, multiple copies may be useful in attaining high levels of gene expression.

Wallroth et al. (in press) have mapped T-DNA inserts in 6 transgenic *Petunia hybrida* plants. While we have not genetically mapped any T-DNA inserts in tomato as yet, we have produced transformed plants of *L. esculentum* x *L. peruvianum* (using either medium D or E) This hybrid is commonly used for mapping purposes in tomato (Tanksley et al, 1984).

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