Theor Appl Genet (1986) 72:770–777

Transformation of cultivated tomato by a binary vector in *Agrobacterium rhizogenes:* transgenic plants with normal phenotypes harbor binary vector T-DNA, but no Ri-plasmid T-DNA

E. A. Shahin, K. Sukhapinda, R. B. Simpson and R. Spivey

ARCO Plant Cell Research Institute, 6560 Trinity Court, Dublin, CA 94568, USA

Received April 14, 1986; Accepted May 20, 1986
Communicated by P. Maliga

Summary. Cultivated tomato was genetically transformed using two procedures. In the first procedure, punctured cotyledons were infected with "disarmed" *Agrobacterium tumefaciens* strain LBA4404 or with *A. rhizogenes* strain A4, each containing the binary vector pARC8. The chimeric neomycin phosphotransferase (NPT II) gene on pARC8 conferred on transformed plant cells the ability to grow on medium containing kanamycin. Transformation reproducible yielded kanamycin-resistant transformants in different tomato genotypes. NPT II activity was detected in transformed calli and in transgenic plants. All of these plants were phenotypically normal, fertile and set seeds. Using the second procedure, inverted cotyledons, we recovered transformed tomato plants from *A. rhizogenes*-induced hairy roots. In this case, all of the transgenic plants exhibited phenotypes similar to hairy root-derived plants reported for other species. Southern blot analysis on these plants revealed that the plant DNA hybridized with both probes representing pARC8-T-DNA, and the T-DNAs of the A4 Ri-plasmid. However, southern analysis on those phenotypically normal transgenic plants from the first procedure revealed that only the pARC8-T-DNA was present in the plant genome, thus indicating that the pARC8-T-DNA integrated into the plant genome independently of the pRi A4-T-DNA. Genetic analysis of these phenotypically normal transgenic plants for the kanamycin-resistance trait showed Mendelian ratios, 3:1 and 1:1, for selfed (R1) and in crossed progeny, respectively.

Key words: *Lycopersicon esculentum* – Transformation – Hairy-root – *A. tumefaciens* – *A. rhizogenes*

Introduction

Agrobacterium tumefaciens is a pathogen of many dicotyledonous crop plants. The bacterium causes a tumor at the infection site of the host plant due to the integration and expression of transferred DNA (T-DNA) from a bacterial plasmid into the host genome (Chilton et al. 1977). Normally, plants do not regenerate from tumors or tissues that contain the T-DNA. In rare cases, however, normal plants have been regenerated from tobacco tumors, but these plants have lost most of the T-DNA in their genome (Yang and Simpson 1981).

To facilitate plant regeneration from transformed tissues, the partial or complete deletion of the T-DNA was sought. This has led to the development of a number of vectors that are efficient in transforming plant cells that were capable of regenerating into whole plants (Fraley et al. 1983; Zambryski et al. 1983; De Block et al. 1984). Two binary vectors (pARC8 and pARC4) have been developed (Simpson et al. 1986) that are similar in design to other binary vectors (Hoekema et al. 1983; de Framond et al. 1983; Bevan 1984; van den Elzen et al. 1985). The vector pARC8 contains the chimeric gene NOS/NPT consisting of the nopaline synthase (NOS) promoter and the coding region for the enzyme neomycin phosphotransferase (NPT). The NOS/NPT selectable marker confers on plant cells the ability to synthesize NPT and thus, to grow in the presence of kanamycin. The vector pARC4 contains the marker nopaline synthase (NOS) which confers on plant cells the ability to synthesize nopaline. We report here that pARC8 in the "disarmed" *A. tumefaciens* strain LBA4404 (Ooms et al. 1982) is useful in obtaining transformed and phenotypically normal tomato plants.

A related pathogen, *A. rhizogenes,* causes a disease in many dicotyledonous plants characterized by transformed hairy roots after one or two T-DNA portions of the Ri-plasmid (TL-DNA and TR-DNA) integrate into the plant genome (Chilton et al. 1982; White et al. 1982). In contrast to *A. tumefaciens*-induced tumors, *A. rhizogenes*-induced hairy roots, in a few species, are capable of regenerating into whole fertile plants containing full length T-DNA (David et al. 1984; Tepfer 1984). As a result of T-DNA presence, abnormal phenotypes

appear in the regenerated plants (David et al. 1984; Tepfer 1984; Costantino et al. 1984; Ooms et al. 1985 a, b).

In this communication, we report the use of the binary vector pARC8 in a virulent strain of *A. rhizogenes* (A4; White and Nester 1980). Two types of transgenic tomato plants were obtained: 1) phenotypically-normal plants which contain pARC8-T-DNA only and 2) plants with hairy root-associated phenotypes which contain both pARC8-T-DNA and the Ri-plasmid T-DNAs.

Materials and methods

Plant materials

Seeds of tomato (*Lycopersicon esculentum* L.) cv. 'Red Cherry', 'Improved Pearson', 'UC-82', 'Heinz 2152' and 'ONT 7710', were provided by Dr. Susan Peters, ARCO Seed Co, Yuba City, California, and Dr. Ben George, Heinz, Tracy, California. The seeds were sterilized with 10% Clorox for 20 min, rinsed twice with sterile distilled water, and then germinated on TM-1 medium (Shahin 1985) in Magenta Boxes (10 seeds per box). The boxes were maintained at 25 °C, 16 h photoperiod, and 4500 lx light intensity.

Agrobacterium strains

Inoculum, from *A. rhizogenes* strain A4 (White and Nester 1980), or the "disarmed" *A. tumefaciens* strain LBA4404 (Ooms et al. 1982), containing the binary vector pARC8, was prepared by growing the bacteria overnight in solid AB medium (Chilton et al. 1974) plus 5 μg/ml tetracycline at 30 °C; the tetracycline selects for the presence of a bacterial marker on the vector. Ten ml of min. A medium (Miller 1972) was added to the culture, and the plates were swirled to resuspend the bacteria at an O.D reading of 0.6–0.8 at 600 nm.

Inverted cotyledon transformation

Individual cotyledons were excised from 8–10 day old 'Red Cherry', and 'H-2152' seedlings using a sharp scalpel which was immersed in a bacterial suspension (*A. rhizogenes*) prior to each use. The excised cotyledons were placed, with their upper surface in contact with the agar, on TM-4G medium, which is the same as TM-4 (Shahin 1985) except sucrose was replaced by 20 g/l glucose. After 7–10 days, hairy roots were excised and cultured on hormone-free TM-4 medium supplemented with 50 mg/l kanamycin and 250 mg/l cefatoxime. For plant regeneration, hairy roots were placed on callus induction medium (Lillo and Shahin 1986) for 2 weeks, then placed on TM-4G medium. Shoots were rooted easily on TM-5 medium (Shahin 1985) or TM-1 medium. In all the above steps, the cultures were kept at 25 °C, 16 h photoperiod, and 2500 lx light intensity.

Punctured cotyledon transformation

Eight–ten day old-seedlings of cvs. 'Heinz 2152', 'Improved Pearson', and 'UC-82' were excised 1-inch beneath the cotyledon and then placed on a sterilized paper towel with the cotyledon upside down. A sterile toothpick or dissecting needle was immersed in the bacterial suspension, and then was used to puncture the cotyledon (8–10 holes). The excised seedlings were transplanted, with the hypocotyl vertically immersed in the agar (in TM-4G medium). The plates were kept under 25 °C, 16 h photoperiod, and 2500 lx light intensity. Two days later, the cotyledons were detached, cut into small pieces (2–3 per cotyledon), and then placed, with the upper surface touching the agar, on selection medium (same as TM-4G except gibberellic acid (GA3) was replaced by 0.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) plus 50 mg/l kanamycin and 500 mg/l cefatoxime). The plates were incubated at the same conditions as before. The kanamycin-resistant calli were removed from the cotyledonary explants and then subcultured on TM-4G medium supplemented with 50 mg/l kanamycin for a second round of selection. Two weeks later, the green calli were removed and subcultured on TM-4G medium (without kanamycin) for further shoot induction and elongation. Shoots were rooted on TM-5 medium. The enzymatic assay for neomycin phosphotransferase (NPT-II) was performed essentially as described by Reiss et al. (1984).

Isolation of tomato DNA and Southern blot analysis

DNA was isolated from leaves of transformed tomato plants as described (Simpson et al. 1986). Southern blot analysis (Southern 1975) was performed essentially as described (Thomashow et al. 1980) using restriction enzymes and a nick translation kit from Bethesda Research Laboratories. The clones of the Ri plasmid T-DNA regions, pFW94 and pFW41, were supplied by Frank White and used as probes to determine the presence of TL-DNA and TR-DNA, respectively (Huffman et al. 1984). The plasmid pNEO105 (Simpson et al. 1986) containing the chimeric gene NOS/NPT was used as the probe of the transferred portion of the pARC8-T-DNA.

Genetic analysis

Transgenic tomato plants (Ro) were self-pollinated as well as crossed to nontransformed plants. The seeds were sterilized with 20% chlorox for 20 min, rinsed with sterile distilled water and then germinated on TM-1 medium (Shahin 1985) in a growth chamber with 2500 lux light intensity and 24 °C temperature. The germinated seedlings were excised 1–2 cm below the cotyledon, and then transplanted into the agar of TM-1 medium (in 25 × 100 mm plates, Falcon) supplemented with 50 mg/l kanamycin plus 50 mg/l cefatoxime. The plates were incubated at the same conditions as above. One week later, the number of root formation was recorded.

Results

Transformation of inverted cotyledon

The excised-ends of cotyledons from tomato cultivars 'Red Cherry' or 'H-2152' were infected with *A. rhizogenes* strain A4 containing the binary vectors pARC8 or pARC4. After 7–10 days, multiple hairy roots developed from the site of infection (Fig. 1 A). In contrast, non-infected cotyledons produced white-snowy callus but did not produce roots. In both cultivars, profuse roots were observed on a high percentage of the inoculated cotyledons (Table 1). Those without roots, exhibited a white-snowy callus which is similar to that in the non- infected cotyledons. The roots were excised, one root per cotyledon, and propagated on hormone-free TM-4 medium supplemented with 50 mg/l kanamycin and 100 mg/l cefatoxime. Most of the roots from infection with strain A4 containing the binary vec-

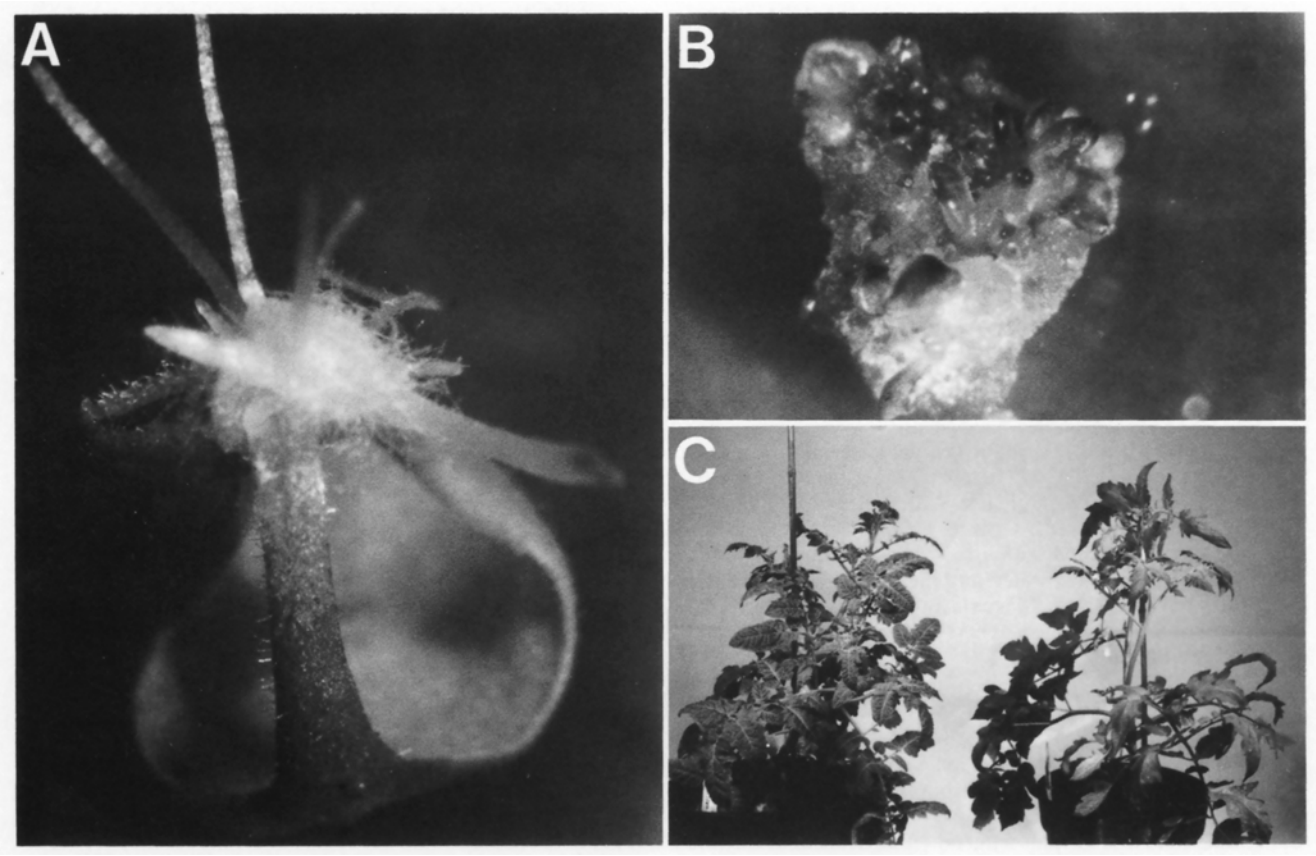

Fig. 1 A–C. Transformation of inverted cotyledon, cv. 'Red Cherry', with *A. rhizogenes.* **A** An inverted cotyledon with multiple hairy roots originating from the inoculation site. **B** Multiple shoot induction from hairy root-derived callus. **C** Hairy root-derived plant exhibiting wrinkling of the leaves (*left*). A normal plant regenerated from non-transformed root (*right*)

Table 1. Hairy root formation on cotyledons induced by *A. rhizogenes* strain A4 containing different binary vectors

Cultivar	Bacterium	Vector	Vector marker	Cotyledons		Kanamycin-resistant roots[a]	NPT-II assay[b]
				Inoculated	With roots		
'Red Cherry'	A4	pARC8	NPT	40	38	35/38	7/7
	A4	pARC8	NOS	40	35	0/35	
	A4	NONE	–	30	26	0/26	
	NONE	–	–	30	0	n.t.[c]	
'H-2152'	A4	pARC4	NOS	64	56	0/56	
	A4	pARC8	NPT	65	58	51/58	9/9
	NONE	–	–	34	0	n.t.[c]	

[a] To avoid siblings, only one root per cotyledon was selected and placed on hormone-free TM-4 medium supplemented with 50 mg/l kanamycin and 100 mg/l cefatoxime

[b] Kanamycin-resistant roots were randomly selected and assayed for the neomycin phosphotransferase activity (NPT-II) as described (Reiss et al. 1984). The table lists the number positive over the number tested

[c] n.t. = not tested

tor pARC8 were resistant to kanamycin (Table 1). The transformed nature of these roots was further confirmed by the activity of NPT-II (Table 1). As expected, roots that were incited with strain A4 containing the binary vector pARC4 did not survive on kanamycin since pARC4 does not contain the NOS/NPT marker. When placed on callus induction medium, tomato hairy roots produced dark-cream slow growing callus along with white-fast growing callus. The latter yielded multiple shoots (Fig. 1 B) when removed and placed on TM-4G medium. As described earlier (Shahin 1985), it is critical to keep dissecting and moving the green callus on TM-4G medium to allow shoot induction. Shoot induction also was achieved when hairy root segments (0.5 cm) were cultured on basal TM-4 medium supplemented with 28.5 μM indoleacetic acid (IAA), 4.45 μM benzyladenine (BAP) and 0.005 M m-inositol. Under these conditions, shoots were observed 4 weeks later arising from callus areas adjoining the root. It is not clear whether the shoot was directly induced from the root or from the root-derived callus. Plant regeneration from these shoots was achieved as described (Shahin 1985).

Transformation of punctured cotyledons

A. rhizogenes cells containing the binary vector pARC8 were introduced into cotyledons wounded with a toothpick. After two days incubation with the *Agrobacterium* to allow infection and plant cell division in vivo, the cotyledons were cut into 2–3 pieces and transferred to a medium containing kanamycin in addition to cefatoxime (to suppress bacterial growth). After 2 weeks, kanamycin-resistant minicalli were observed at the wound site (Fig. 2A). A high proportion of the wound sites contained calli that were resistant to kanamycin (Table 2). Control wounds inoculated with *A. rhizogenes* strain A4 containing pARC4, a vector lacking the NPT II gene, produced no calli around the holes. Most but not all of the calli from the experiment with pARC8 were resistant to a second round of selection on kanamycin (Table 2). Prior to shoot induction, the nature of the transformed calli was confirmed again by the NPT-II activity (Fig. 2 B). Shoot induction was achieved by further subculturing on TM-4G medium without kanamycin. Since we do not know the clonal nature of the transformed callus, we selected only one shoot per wound as an independent transformant. Phenotypically-normal and fertile plants were regenerated from three cultivars and grown to maturity in the greenhouse.

Puncturing the cotyledon with a tooth pick contaminated with the "disarmed" *A. tumefaciens* strain LBA4404 containing the binary vector pARC8 resulted in kanamycin-resistant calli at a frequency similar to that obtained with *A. rhizogenes* (Table 2). The process of regeneration was also similar, except the kanamycin-resistant calli developing from *A. tumefaciens* infection appeared about two weeks later.

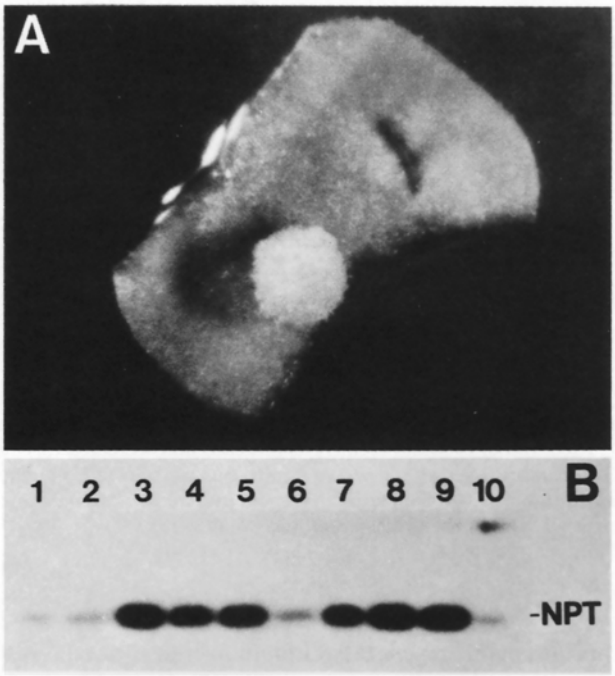

Fig. 2A, B. Transformation of punctured cotyledons with *A. rhizogenes.* **A** Selection of transformed mini-calli for kanamycin resistance; punctured cotyledons transformed with the binary vector pARC8 in *A. rhizogenes* were grown on a medium supplemented with 50 mg/l kanamycin (see "Materials and methods"). **B** Neomycin phosphotransferase II assay (Reiss et al. 1984) of randomly selected kanamycin-resistant calli. Numbers 1–9 represent 9 samples randomly selected kanamycin-resistant green calli from cv. 'H2152', and sample No. 10 is a positive control (bacteria with the transposon Tn5). The exposure time was 2 h

Phenotype of the regenerated plants

From 3 independent hairy root cultures, of cv. 'Red Cherry', 58 shoots were regenerated and grown to maturity in soil. NPT-II assays performed on leaves from 7 plants in the greenhouse were all positive (data not shown). Although the transformed tomato plants appeared normal during development in agar plates and potted plants grew just as vigorously (Fig. 1 C), the transformed plants differed in several ways from non-transformed, root-derived tomato plants once they were in soil. The leaves of transformed plants were wrinkled to different degrees. Pollen viability, assayed by germination in vitro, was reduced and may be the cause of the observed reduction in fruit and seed set. Furthermore, the size of the flower and fruit were reduced. The roots of the transformed plants appeared to lack apical dominance as evidenced by an increased lateral root production. Root plagiotropism was also observed. In

Table 2. Transformation of tomato punctured cotyledons using the binary vector pARC8 in *A. rhizogenes* strain A4 or in the disarmed *A. tumefaciens* strain LBA4404

Cultivar	Bacterium/vector	Punctured cotyledons	Kanamycin-resistant calli		No. of transgenic plants[b]
			1st selection[a]	2nd selection[a]	
'Improved Pearson'	A4/pARC8	120	96	83	(65)
'Heinz 2152'	A4/pARC8	320	270	145	(53)
'UC-82'	A4/pARC8	45	39	30	(13)
'ONT7710'	LBA4404/pARC8	61	11	9	(n.t.)[c]
'UC-82'	LBA4404/pARC8	147	128	101	(45)
'Heinz 2152'	LBA4404/pARC8	58	36	27	(23)

[a] Control wounds inoculated with *A. rhizogenes* strain A4 containing pARC4, a vector lacking the neomycin phosphotransferase gene, produced no calli around the holes

[b] The number of kanamycin-resistant calli that were actually regenerated into plants. Multiple shoots (3 and more) were obtained per calli, but we selected one shoot per calli to represent independent transformants. It was possible to regenerate all the kanamycin-resistant calli by continuous subculturing on TM-4G medium as described (Shahin 1985)

[c] n.t. = not tested

contrast, plants regenerated from non-transformed roots of cv. 'Red Cherry' were phenotypically normal for leaf, fruit and root characteristics.

On the other hand, transgenic plants obtained from calli via punctured cotyledon (transformed by either *A. tumefaciens* or *A. rhizogenes*) were of normal phenotype similar to those obtained from non-transformed cotyledon (Fig. 2C). The flowers were normal, fertile and set fruits with abundant seeds.

Foreign DNA in tomato plants

Vector DNA from pARC8, or its derivative pARC16, was directly demonstrable by Southern blot analysis of the genomes of transformed tomato plants obtained from inverted cotyledons and from punctured cotyledons (Fig. 3A). Bands corresponding to fragments at the border between the vector DNA and plant DNA were evident from a hairy root-derived plant (lane 3). Since the probe was homologous to both borders and there were two such bands, there was probably one copy of the T-DNA in this plant. A phenotypically-normal plant, containing pARC16 from a punctured cotyledon inoculated with *A. rhizogenes* containing pARC16, probably contained two copies of T-DNA in tandem (lane 4) since there appeared to be two bands that correspond to the left and right borders, respectively, plus a band which was roughly twice the intensity of the former bands and which has a mobility expected for a left border fragment joined to a right border fragment (about 7.2 KB). In a similar manner, we estimated that in two independent plants there was one copy of DNA transferred from a vector in the disarmed *A. tumefaciens* strain LBA4404 (lanes 5 and 6). No bands were visible in controls from untransformed plants (lane 1 and 2).

Only transformed plants derived from a hairy root contained what appeared to be a complete TL-DNA and some portions of the TR-DNA of the Ri-plasmid. Lane 3 in Fig. 3B shows bands with the expected mobilities (4.2, 3.4, 1.8 and 1.6 KB; Huffman et al. 1984) for the internal Hind III fragments of the TL-DNA of the A4 Ri plasmid (3B, C). Lane 3 in Fig. 3C shows that although there was no band with mobility expected of the internal Hind III fragment (5.1 kb; Huffman et al. 1984), there was clearly some portion of the TR-DNA of the A4 Ri plasmid in the plant derived from a hairy root. In contrast, a phenotypically-normal plant derived from the punctured cotyledon method contained no detectable Ri plasmid DNA (Fig. 3B and C, lane 4) even though they originated from an inoculation with the *A. rhizogenes* strain A4. As expected, no TL or TR-DNA were found in the control plants (Fig. 3B and C, lanes 1 and 2) or in LBA4404-transformed plants (Fig. 3B and C, lanes 5 and 6). Copy number reconstruction experiments (not shown) indicated that less than a half of a copy of TL or TR-DNA would have been detectable.

Inheritance of kanamycin resistance

Table 3 presents the genetic segregation of the kanamycin-resistance trait, characterized by the ability of shoots (excised from seedlings) to root in the presence of kanamycin. These data are consistent with the ratios 3 resistant to 1 sensitive that are expected from self-fertilization of a heterozygous individual in the case in which resistance is conferred by a dominant allele of a single nuclear gene. Since these transgenic plants were derived from diploid tomato plants ($2n=24$), they would be expected to be heterozygous for a dominant allele. Additional evidence for Mendelian inheritance is

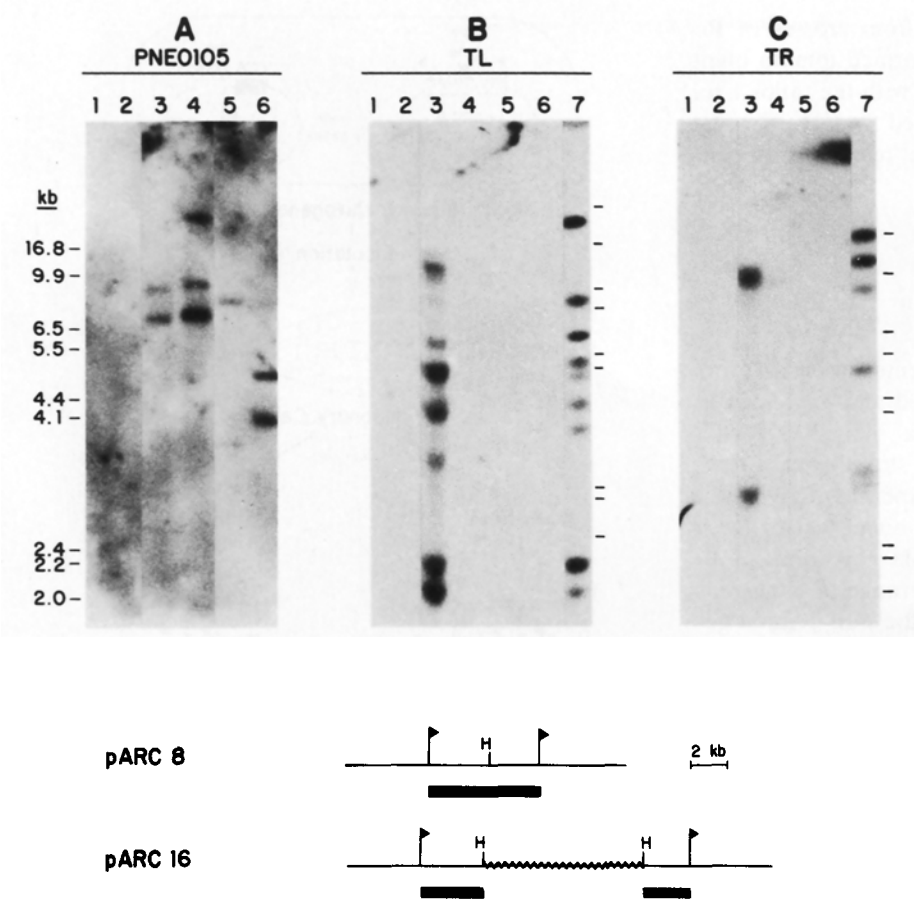

Fig. 3. (*Top*). Southern blot analysis of DNA from transformed tomato plants. The probes were as follows: (*Panel A*) pNEO105, which contains the NOS/NPT marker found in the vectors pARC8 and pARC16 (Simpson et al. 1986); (*Panel B*) pFW94 (Huffman et al. 1984), which contains the entire TL-DNA from the Ri plasmid from *A. rhizogenes* strain A4; (*Panel C*) pFW41 (Huffman et al. 1984), which contains the entire TR-DNA from the Ri plasmid in *A. rhizogenes* strain A4. The blots represent Hind III digests of 5 μg DNA from the following sources: (*Lane 1*) Non-transformed cv. 'VF-36' plant; (*Lane 2*) Plant regenerated from untransformed cv. 'VF-36' root; (*Lane 3*) Hairy root-derived plant from tissue (cv. 'Red Cherry') transformed by strain A4 containing the vector pARC16; (*Lane 4*) Phenotypically-normal plant obtained from transforming punctured cotyledon (cv. 'Improved Pearson') with strain A4 containing pARC16; (*Lanes 5 and 6*) independent transgenic plants from punctured cotyledons (cv. 'UC-82B') transformed by disarmed *A. tumefaciens* strain LBA4404 containing the vector pARC8; (*Lane 7*) Internal stadards, pFW94 (*Panel B*) or pFW41 (*Panel C*). At the bottom of the figure are drawings of the expected maps for a single insert of transferred DNA (between the flags) from pARC8 and from pARC16, a derivative of pARC8 with an insert in the Hind III site. The *solid bar* indicates the regions homologous to the probe pNEO105 and the *H* marks the locations of Hind III sites

Table 3. Inheritance of kanamycin resistance of randomly selected transgenic tomato plants transformed via the punctured cotyledon method

Cross(line)	Bacterium/vector	Total number of seedlings tested[a]	Kanamycin-resistant seedlings[b]	Kanamycin-sensitive seedlings[c]	Ratio	x^2	*P*
435-81-13×H1916	A4/pARC8	47	25	22	1:1	0.18	0.50–0.80
435-81-25×H1916	A4/pARC8	60	30	30	1:1	0	
435-81-48×H1916	LBA4404/pARC8	44	27	17	1:1	2.27	0.05–0.20
435-81-52×H1916	LBA4404/pARC8	42	23	19	1:1	0.38	0.50–0.80
435-81-16 (selfed)	A4/pARC8	21	14	7	3:1	1.77	0.20–0.50
435-81-25 (selfed)	A4/pARC8	69	49	20	3:1	0.58	0.20–0.50
435-81-18 (selfed)	A4/pARC8	39	29	10	3:1	0.008	0.80–0.95
435-81-32 (selfed)	A4/pARC8	58	39	19	3:1	1.86	0.05–0.20
435-81-48 (selfed)	LBA4404/pARC8	55	40	15	3:1	0.15	0.50–0.80
435-81-52 (selfed)	LBA4404/pARC8	71	52	19	3:1	0.12	0.50–0.80

[a] One week old seedlings were excised just below the cotyledon and then transplanted into the agar of TM-1 medium (see "Materials and methods) supplemented with 50 mg/l kanamycin plus 50 mg/l cefatoxime

[b] Transgenic tomato seedlings, containing the NOS/NPT selectable marker produced roots in the presence of 50 mg/l kanamycin. This was confirmed by the NPT-II assay and Southern blot analysis (data not shown)

[c] Sensitive seedlings produced no roots on kanamycin-supplemented TM-1 medium (50 mg/l). However, within a week, the green color disappeared and the seedlings turned violet (due to heavy pigmentation) and eventually withered and died without developing a primary leaf

provided by the results obtained from crosses of Ro transgenic plants with a non-transformed tomato plant (Table 3). These data are consistent with the ratios 1 resistant to 1 sensitive that are expected from crosses of a heterozygous individual where the resistance is conferred as single dominant.

Discussion

The data presented in this paper demonstrate that foreign DNA can be readily transferred and expressed in regenerated tomato plants using a binary vector in either a "disarmed" *A. tumefaciens* strain or in a virulent *A. rhizogenes* strain. In these experiments, we used the binary vector pARC8, but other binary vectors with a selectable marker should be suitable. The ability to regenerate whole plants from a wide range of tissues including protoplasts (Shahin 1985), the well-defined genetics of tomato (Rick 1975), plus the simple and convenient transformation systems described here make this crop plant fully amenable to the modern methods of plant molecular genetics.

We have used *A. rhizogenes* plus the binary vector pARC8 in two different transformation procedures that resulted in two types of transgenic tomato plants. The punctured cotyledon procedure, permitted the selection of kanamycin-resistant calli and shoots which regenerated into normal plants that contained vector T-DNA. In contrast, the inverted cotyledon procedure using the same bacteria resulted in hairy roots which regenerated into plants with abnormal phenotypes that contained, in addition to the vector T-DNA, the Ri-plasmid T-DNAs. Because of the frequent presence of vector T-DNA in the hairy roots (Table 1), a binary vector such as pARC4 (with the nopaline synthase gene, a marker for which there is a screen but no selection) was also useful in this latter procedure.

The two alternative gene transfer systems (via hairy roots from inverted cotyledons or via callus from punctured cotyledons) provide a choice for the molecular biologist in the genetic manipulation of tomato. The recovery of phenotypically-normal transgenic plants via the punctured cotyledon procedure using either a "disarmed" *A. tumefaciens* or virulent *A. rhizogenes* makes it suitable for introducing a single gene (e.g. disease resistance) into an already adapted variety where no other genic modifications are desired. On the other hand, the *A. rhizogenes* (using the inverted cotyledon procedure) can be used to introduce foreign genes into tomato plants which also possess the hairy root-associated phenotypes. This may provide an opportunity for using this system in crop plants where the regeneration from roots is the only avenue to genetic manipulation, or in situations where some hairy root-associated phenotypes (e.g. shallow, extensive root systems) are desirable.

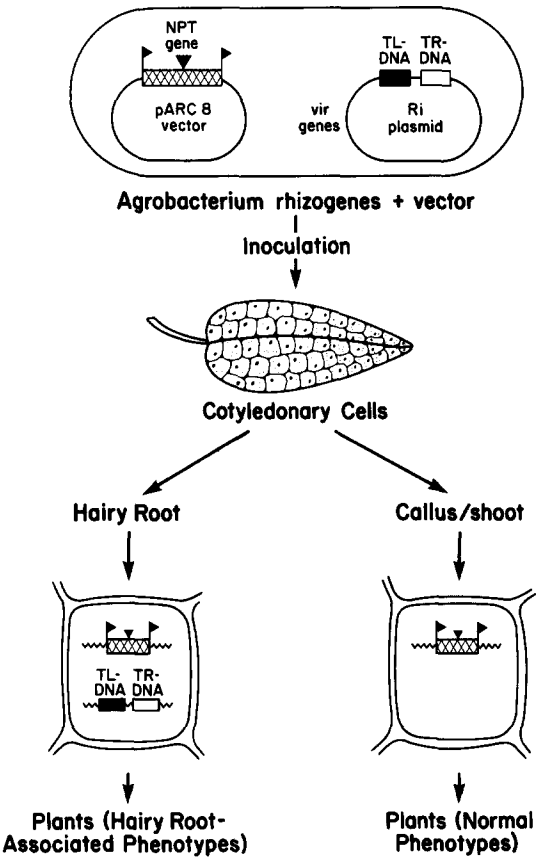

Fig. 4. Schematic diagram of the two procedures used to transform tomato cotyledons by a virulent *A. rhizogenes* strain A4 containing the binary vector pARC8 Although the same bacterium was used in each procedure, two types of transgenic plants were obtained: 1) Inoculation of the excised-end of the cotyledon resulted in hairy roots which gave rise to transgenic plants with hairy-root associated phenotypes, which contain both vector DNA and Ri-plasmid T-DNAs and 2) puncturing the cotyledon yielded kanamycin-resistant calli that produced phenotypically-normal tomato plants which contain vector DNA only

Since *Agrobacterium rhizogenes* containing two distinct T-DNAs (on the Ri-plasmid and on a binary vector) can transfer one, or both to a plant cell (Fig. 4), depending on the procedures used, binary vectors may be used in other virulent strains of *Agrobacterium* to produce normal, transgenic plants. For example, we have recently used inverted tomato cotyledons (cv. 'UC-82B') with pARC8 in *A. tumefaciens* strain A328, a shoot-inducing mutant (Garfinkel et al. 1981) of the pTi A6 plasmid. Both tumors and shoots at the inoculation site exhibited NPT activity. Additionally, phenotypically-normal and fertile plants were regenerated from these transformed shoots (unpublished results). This model experiment might be extended to cover other host

plants, particularly those that are difficult to transform and regenerate such as soybean and other legumes. Furthermore, new mutants of *Agrobacterium* might be constructed which will help regeneration in other species.

The use of aseptic cotyledonary tissue for *Agrobacterium* inoculations is desirable because the tissue is easy to obtain. Furthermore, cotyledonary cells may have a high potential for regeneration as suggested by the recent success in regenerating plants from cotyledon-derived protoplasts of previously nonamenable crops (Davey 1983).

Acknowledgements. We thank Drs. Simon Bright, Jack Erion, Jon Fobes and Valerie Williamson for their critical review of the manuscript; Mark Staebell for the photographic work; Mayer Yashar for excellent tecnhical assistance; Linda Margossian for help with the DNA analysis; Karen Long for typing the manuscript and Dale Cetlinski for the art work.

References

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

Chilton M-D, Currier TC, Farrand SK, Bendich AJ, Gordon MP, Nester EW (1974) *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumors. Proc Natl Acad Sci USA 71:3672–3676

Chilton M-D, Drummond MD, Merlo DJ, Sciaky D, Montoya AL, Gordon MP, Nester EW (1977) Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. Cell 11:263–271

Chilton M-D, Tepfer D, Petit A, David C, Casse-Delbart F, Tempe' J (1982) *Agrobacterium rhizogenes* inserts T-DNA into the genomes of the host plant root cells. Nature 295:432–434

Costantino P, Spano L, Pomponi M, Benvenuto E, Ancora G (1984) The T-DNA of *Agrobacterium rhizogenes* is transmitted through meiosis to the progeny of hairy root plants. J Mol Appl Genet 2:465–470

David C, Chilton M-D, Tempe' J (1984) Conservation of T-DNA in plants regenerated from hairy root cultures. Biotechnology 2:73–76

Davey MR (1983) Recent developments in the culture and regeneration of plant protoplasts. Experienta (Suppl) 46:19–29

De Block M, Herrera-Estrella L, Van Montagu M, Schell J, Zambryski P (1984) Expression of foreign genes in regenerated plants and in their progeny. EMBO J 3:1681–1689

de Framond AJ, Barton KA, Chilton M-D (1983) Mini-Ti: A new vector strategy for plant genetic engineering. Biotechnology 1:262–269

Fraley RT, Rogers SG, Horsch RB, Sanders PR, Flick TS, Adams SP, Bittner ML, Brand LA, Fry JS, Gallupi GR, Goldberg SB, Hoffman NL, Woo SC (1983) Expression of bacterial genes in plant cells. Proc Natl Acad Sci USA 80:4803–4806

Garfinkel DL, Simpson RB, Ream LW, White FF, Gordon MP, Nester EW (1981) Genetic analysis of crown gall: fine structure map of the T-DNA by site-directed mutagenesis. Cell 27:143–153

Hoekema H, Horsh PR, Hooykaas PJJ, Shilperoort RA (1983) A binary plant vector strategy based on separation of vir- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature 303:179–181

Huffman GA, White FF, Gordon MP, Nester EW (1984) Root-inducing plasmid: physical map and homology to tumor-inducing plasmids. J Bacteriol 157:269–276

Lillo C, Shahin EA (1986) Rapid regeneration of plants from hypocotyl protoplasts and root segments of cabbage. Hort Science 21:315–317

Miller JH (1972) Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor NY, pp 432–433

Ooms G, Hooykaas PJJ, Van Veer RJM, Van Bellen P, Regensburg-Tunik TJG, Schilperoort RA (1982) Octopine Ti-plasmid deletion mutants of *Agrobacterium tumefaciens* with emphasis on the right side of the T-region. Plasmid 7:15–29

Ooms G, Karp A, Burrell MM, Twell D, Roberts J (1985a) Genetic modification of potato development using Ri Ti-DNA. Theor Appl Genet 70:440–446

Ooms G, Bains A, Burrell M, Twell D, Wilcox E (1985b) Genetic manipulation in cultivars of oilseed rape (*Brassica napus*) using *Agrobacterium*. Theor Appl Genet 71:325–329

Reiss B, Sprengel R, Will H, Schaller H (1984) A new sensitive method for qualitative and quantitative assay of neomycin phosphotransferase in crude cell extracts. Gene 30:217–223

Rick CM (1975) The tomato. In: King R (ed) Handbook of genetics, vol 2. Plenum Press, New York, pp 247–280

Shahin EA (1985) Totipotency of tomato protoplasts. Theor Appl Genet 69:235–241

Simpson RB, Spielmann A, Margossian L, McKnight TD (1986) A disarmed binary vector from *Agrobacterium tumefaciens* functions in *Agrobacterium rhizogenes:* frequent co-transformation of two distinct T-DNAs. Plant Mol Biol 6:403–415

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517

Tepfer D (1984) Transformation of several species of higher plants by *Agrobacterium rhizogenes:* sexual transmission of the transformed genotype and phenotype. Cell 37:959–967

Thomashow MF, Nutter R, Montoya A, Gordon M, Nester EW (1980) Integration and organization of Ti plasmid sequences in crown gall tumors. Cell 19:729–739

van den Elzen PJM, Townsend J, Lee KY, Bedbrook JR (1985) A chimeric hygromycin resistance gene as a selectable marker in plant cells. Plant Mol Biol 5:299–302

White FF, Nester EW (1980) Molecular and genetic analysis of the transferred DNA regions of the root inducing plasmid of *Agrobacterium rhizogenes.* J Bacteriol 141:1134–1141

White FF, Ghidossi G, Gordon MP, Nester EW (1982) Tumor induction by *Agrobacterium rhizogenes* involves the transfer of plasmid DNA to the plant genome. Proc Natl Acad Sci USA 79:3193–3197

Yang F, Simpson RB (1981) Revertant seedlings from crown gall tumors retains a portion of the bacterial Ti plasmid DNA sequences. Proc Natl Acad Sci USA 78:4151–4155

Zambryski P, Joos H, Genetello C, Leemans J, Van Montagu, Schell J (1983) Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. EMBO J 2:2143–2150