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Genetics

Analysis of *Agrobacterium tumefaciens* virulence mutants in leaf discs

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ABSTRACT **The leaf disc transformation system provides a simple means to score expression of various T-DNA markers within days of infection by *Agrobacterium tumefaciens* as well as long-term selection for growth of transformed callus and shoots. In this report, we describe the application of this system to evaluation of marker transfer and integration in a comprehensive set of defined avirulent mutants of *A. tumefaciens*. We conclude that *virC* is not essential when the T-DNA is present on a binary vector. The most likely explanation for this is that the *virC* gene product is involved in generation of a T-DNA intermediate.**

Agrobacterium tumefaciens is the causative agent of crown gall disease, a neoplastic transformation of plant cells (reviewed in refs. 1–3). Ability to form tumors is conferred by a large extrachromosomal element, the Ti (tumor-inducing) plasmid. Two regions of the Ti plasmid are crucial for tumor formation. The first of these, the T-DNA, is transferred to a plant cell where it is stably integrated into plant nuclear DNA. The T-DNA is bounded by two direct 25-nucleotide repeats that are involved in transfer to the plant cell. These sequences are also the sites of recombination between bacterial and plant DNA (4–7).

Most of the genes responsible for T-DNA transfer are localized in an ≈35-kilobase (kb) region distinct from the T-DNA, the *vir* region. The *vir* region has been characterized extensively by transposon mutagenesis and at least six operons important for tumorigenesis have been identified (refs. 8, 9; S.S., unpublished data). Although a great deal is known about the location and expression of these operons, little is known about the functions of the gene products. In fact, little is known about the initial events of T-DNA transfer and integration. Recently, a possible intermediate in the transfer has been identified (10). This intermediate appears to be formed in the bacterium by excision and recircularization of the T-DNA at the border sequences, leaving a smaller circular molecule containing a single copy of the border sequence.

We have been interested in elucidating the pathway of T-DNA transfer and integration and determining whether we can separate transfer from integration. The development of a strong and sensitive selectable marker as an alternative to the T-DNA oncogenes combined with the high-frequency leaf disc transformation system provide a powerful tool to analyze the initial events that ultimately lead to tumorigenesis.

The leaf disc transformation system provides a simple means to score expression of various T-DNA markers within days of infection by *A. tumefaciens* as well as allowing long-term selection for growth of transformed callus and shoots (11). In this report, we describe the application of this system to evaluation of marker transfer and integration in a comprehensive set of defined *vir* region mutants of *A. tumefaciens*.

MATERIALS AND METHODS

Plasmid Constructions. Two plasmids were constructed for the purpose of introducing the nopaline synthase and neomycin phosphotransferase genes into *Agrobacterium vir* mutants. The first plasmid, pMON501, is a binary vector and the second, pMON510, is designed to integrate into a resident T-DNA through a region of limited internal DNA homology. The pMON501 vector was constructed by insertion of a 2.4-kb *Bcl* I fragment from pMON200 (11) containing the entire nopaline synthase gene into the unique *Bam*HI site of the binary vector pEND4K (12). The pMON510 vector was constructed by insertion of a 1.4-kb *Bam*HI fragment from pUC4K (13) containing a bacterial kanamycin-resistance gene into the unique *Bgl* II site of pMON200.

***Agrobacterium* Strain Construction.** A representative sampling of *Agrobacterium vir* mutants was selected for testing ability to transform petunia leaf discs. At least two independent mutants in each identified *vir* locus (*A, B, C, D, E,* and *G*) were tested. The mutants contain a modified Tn*3* (14) that has been introduced into pTiA6 by marker exchange. The generation and analysis of these mutants will be described elsewhere. The plasmids pMON501 and pMON510 were introduced into each *vir* mutant by the triparental mating procedure (15). Transconjugants were selected on AB plates (16) containing 200 μg of carbenicillin per ml and 100 μg of kanamycin per ml.

Leaf Discs. The basic leaf disc transformation system has been described (17). The details of the optimized procedure for high-frequency transformation will be described elsewhere. Leaf discs of *Petunia hybrida* (Mitchell doubled haploid) were precultured for 2 days on medium 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% agar. The discs were then soaked (for only a few seconds) in an overnight culture of *A. tumefaciens*, blotted dry, and placed upside-down on nurse culture plates of the same medium (18). After 3 days of coculture, the discs were transferred to the same medium containing 500 μg of carbenicillin per ml and either 0.5 mM arginine for enhancement of opine production or 300 μg of kanamycin per ml for selection of transformed callus.

Opine Detection. After 2 days on medium containing arginine, individual leaf discs were blotted dry, frozen in liquid nitrogen, crushed in Eppendorf tubes, and centrifuged in a Microfuge for 5 min. Four-microliter samples of supernatant were assayed for nopaline content by the method of Otten and Schilperoort (19).

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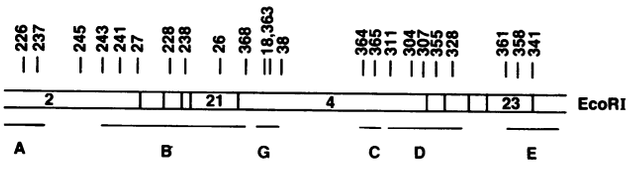
Abbreviation: kb, kilobase(s).



Locus	Phenotype
A	avirulent
B	avirulent
C	weakly virulent (cis) virulent (trans)
D	avirulent
E	weakly virulent
G	avirulent

FIG. 1. *Eco*RI restriction map of the virulence loci of pTiA6 (S.S. and E.W.N., unpublished data). The approximate limits of each locus are indicated. Numbers above the map show the locations of *vir* mutants used in this study.

RESULTS

The virulence region of pTiA6 has been mapped extensively by transposon mutagenesis (refs. 8, 9; S.S., unpublished data). A minimum of six operons have been identified that affect virulence (Fig. 1). We have utilized a series of 24 of these transposon mutants covering each of the six operons as well as wild-type insertion mutants in this region to look for alterations in the ability to transfer and/or integrate T-DNA in a petunia leaf disc transformation system.

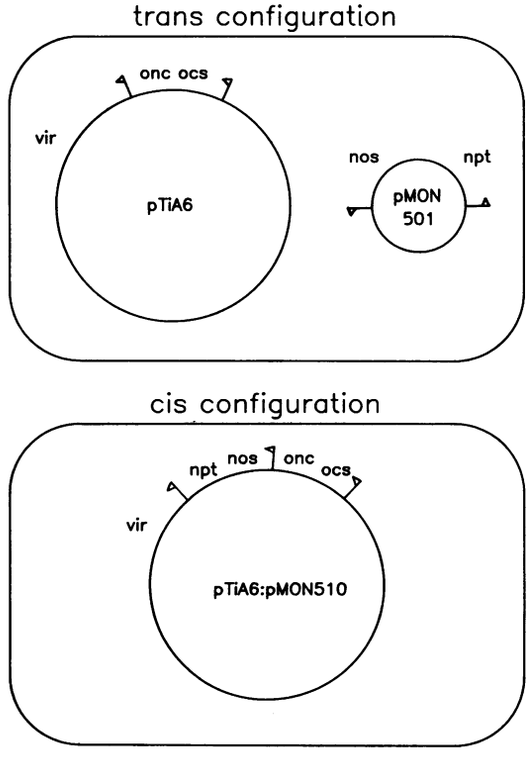


FIG. 2. (*Upper*) Schematic representation of the binary vector system used to assay transformation in *vir* mutants. The 25-base-pair border sequences delimiting the T-DNA are indicated (Δ). The pMON501 plasmid contains the nopaline synthase (*nos*) and neomycin phosphotransferase (*npt*) gene within the T-DNA. The pTiA6 plasmid contains the octopine synthase (*ocs*) and oncogenes (*onc*) within the T-DNA. (*Lower*) *cis* configuration of T-DNA resulting from cointegration of pMON510 into pTiA6. The *nos* and *npt* genes are contiguous with the normal pTiA6 T-DNA.

A new binary vector plasmid, pMON501, was introduced into all of the mutants and the parental A348 strain, creating the double T-DNA configuration shown in Fig. 2. The binary vector, pMON501, contains the nopaline synthase gene (*nos*) and a chimeric gene for kanamycin-resistance, neomycin phosphotransferase (type II, *npt* II). The resident wild-type T-DNA on pTiA6 contains the octopine synthase gene (*ocs*) and the phytohormone biosynthesis genes (*onc*) that cause abnormal growth of transformed cells. The *onc* genes confer the ability to grow in the absence of exogenous phytohormones to transformed plant cells.

All four markers could be assayed and/or selected in the leaf disc transformation system. Opines could be detected within 36 hr of infection and reached a high level by the fifth day after infection. Kanamycin-resistant callus could be observed within 5 days of transfer to selective medium with our standard vector GV3111SE:pMON200 (11) as well as with virulent strains of A348 containing pMON501. Growth of callus on hormone-free medium could be observed within 7 days after transformation with strain A348.

Table 1 summarizes the results for all of the strains tested in the leaf disc system. All *virA*, *-B*, *-D*, and *-G* mutants were completely negative for all four markers of transformation, presumably because the T-DNA never entered the plant cells in these cases. All three *virE* mutants were able to transfer and integrate the markers but only at an extremely attenuated frequency. The opine expression was near the limit of detectability in this assay but could be consistently observed above background. Kanamycin-resistant colonies appeared on one-third to one-half of the infected discs, usually only one or two colonies per disc (Fig. 3). No galls ever formed on

Table 1. Leaf disc transformation with *vir* mutants

vir	Number	Octopine	Nopaline	Kanamycin resistance
A	226	−	−	−
	237	−	−	−
B	26	−	−	−
	27	−	−	−
	228	−	−	−
	238	−	−	−
	241	−	−	−
	243	−	−	−
	368	−	−	−
C	364	<+	++	++
	365	<+	++	++
D	304	−	−	−
	307	−	−	−
	311	−	−	−
	328	−	−	−
	355	−	−	−
E	341	<+	<+	<+
	358	<+	<+	<+
	361	<+	<+	<+
F	219	+++	+++	+++
G	19	−	−	−
	363	−	−	−
Wt	38	+++	+++	+++
Wt	245	+++	+++	+++

Detection of opines and kanamycin-resistant callus in leaf discs infected with derivatives of pTiA6:pMON501. Weak opine spots (<+) were barely above background but could be reproducibly observed in *virC* and *virE* infected discs and were never observed in the other *vir* mutants. Strong opine spots (+++) or moderate opine spots (++) are illustrated in Fig. 4. High-frequency (+++) and low-frequency (<+) kanamycin resistance is illustrated in Fig. 3. Moderate-frequency (++) kanamycin resistance means that every disc produced numerous callus colonies but not in a confluent ring as with wild-type (Wt) vectors.

petunia stems inoculated with *virE* mutants. Thus, markers on both T-DNAs (*cis* and *trans*) were transferred at a very low frequency to plant cells infected by the *virE* mutants.

The behavior of *virC* mutants was strikingly different: the binary vector markers (*nos* and *npt* II) were transferred at much higher frequency than the Ti plasmid markers (*ocs* and *onc*). Fig. 4 shows the relative amounts of octopine and nopaline produced in these leaf discs. The frequencies of kanamycin-resistant colonies and hormone autonomous colonies were in similar proportions (data not shown).

To test whether the differences in marker transfer frequency were due to the position (*cis* or *trans* configuration relative to the Ti plasmid) or due to a peculiar feature of pMON501, the plasmid was modified slightly to permit integration into the T-DNA of the Ti plasmid. This derivative, pMON510, was then inserted into the *virC* mutants creating the configuration shown in Fig. 2 *Lower*. Although it does not reproduce well in Fig. 4, both octopine and nopaline could be consistently observed in leaf discs infected with *virC* mutants containing pTiA6:pMON510. Kanamycin-resistant callus was also observed at a very low frequency (data not shown). Thus, *virC* mutants appeared to transfer markers on the binary T-DNA at frequencies approaching wild type, whereas the same markers integrated in *cis* into the T-DNA of a Ti

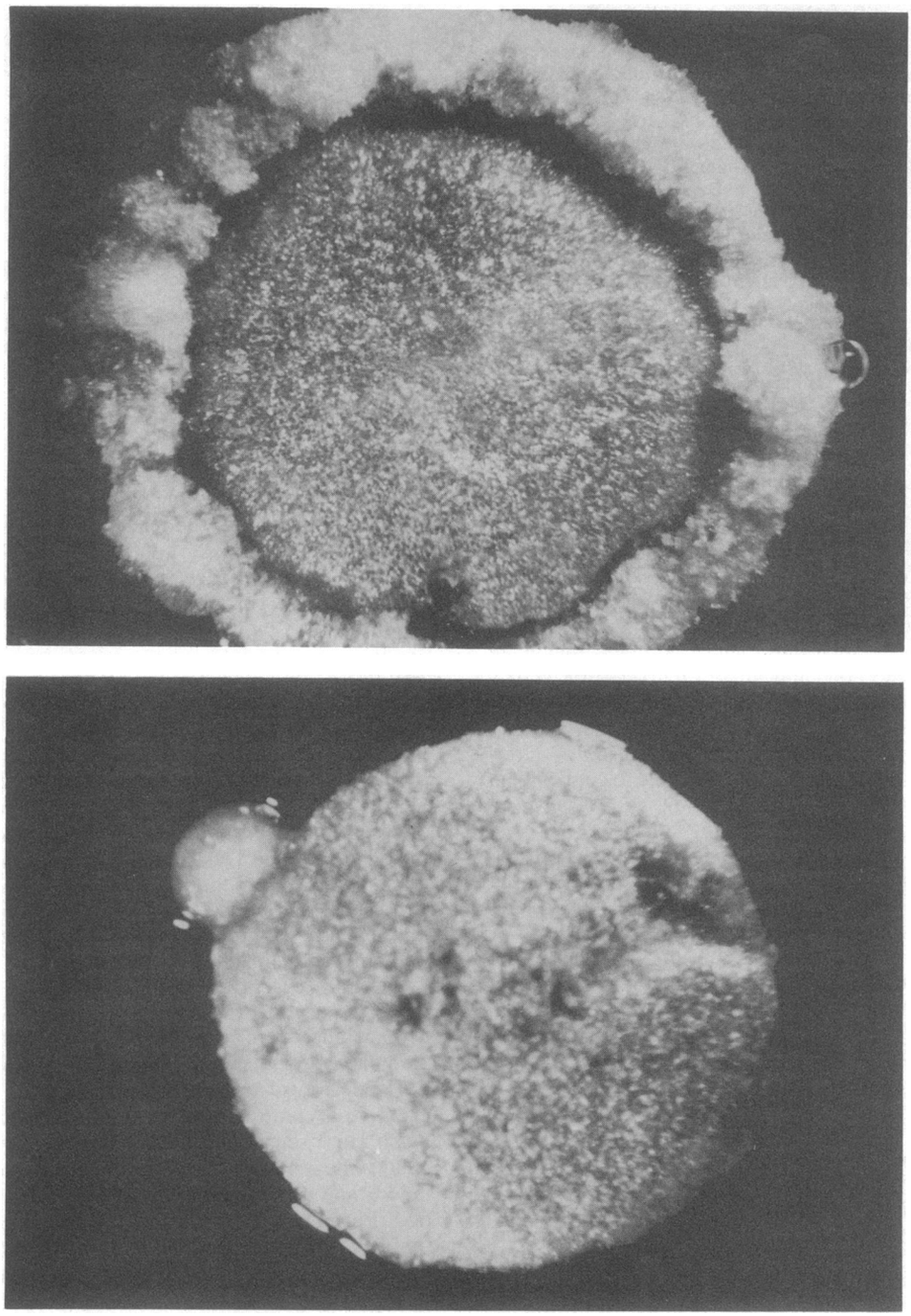


FIG. 3. Kanamycin-resistant callus from leaf discs transformed with A348:pMON501 (*Upper*) or *virE* mutant 358 with pMON501 (*Lower*). Leaf discs were transformed and grown on medium containing 300 μg of kanamycin per ml. The discs infected with the virulent A348:pMON501 strain showed immediate growth and the photograph was taken 2 weeks after transfer to selective medium. The discs infected with the *virE* mutants often showed no growth for the first 2 weeks when tiny sectors became visible. The photograph of the *vir* treated disc was taken 4 weeks after transfer to selective medium. (×10.)

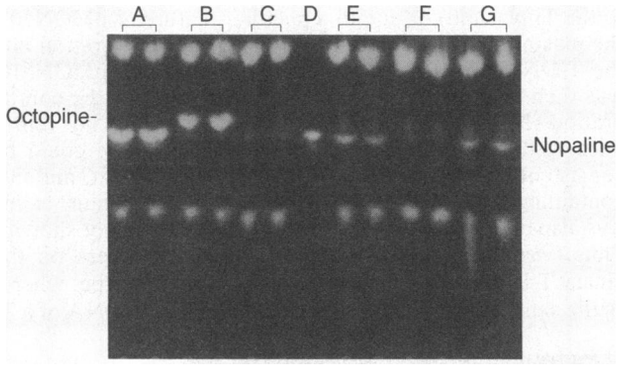


FIG. 4. Opine assay showing the difference in nopaline accumulation in *virC* mutants containing pMON501 (*trans*) or pMON510 (*cis*). Leaf discs were transformed and assayed for nopaline content 5 days after infection by grinding individual discs and analyzing a 4-μl sample of the liquid by paper electrophoresis. There is very little octopine in discs infected with *virC* mutants and this does not convincingly reproduce in a photograph. Duplicate samples for discs infected with *A. tumefaciens* strains containing plasmids are as follows. A, pTiB6S3SE:pMON200. B, pTiA6. C, pTiA6 (*virC* 365): pMON510. D, nopaline standard. E, pTiA6 (*virC* 365):pMON501. F, pTiA6 (*virC* 364):pMON510. G, pTiA6 (*virC* 364):pMON501.

plasmid were transferred at a greatly reduced frequency. No other *vir* mutants or the wild-type controls showed this *cis/trans* bias in transfer of T-DNA markers.

DISCUSSION

The leaf disc transformation system permits simple, efficient, and reproducible examination of *A. tumefaciens* mediated gene transfer into cells in the disc. Whole discs provide a convenient unit for replicating treatment samples and for automatic pooling and averaging of many potentially independent events among the many thousands of cells in a disc. The method permits much more sensitive measurement of gene transfer, expression, and integration than the widely used gall-formation assay on plant stems or leaves. Thus, using the leaf disc system we are able to observe weak transfer from *virE* mutants and differential transfer of different T-DNAs in *virC* mutants. We are also able to confirm that the other *vir*-region mutants (*virA, -B, -D,* and *-G*) give no detectable transfer or integration of the T-DNA.

None of the *vir*-region mutants appeared to cause dissociation of DNA transfer from DNA integration, as measured by early expression of opine production and subsequent stable transformation assays. In every case the presence or absence and the relative ratio of opine production to the linked selectable marker (hormone autonomy or kanamycin resistance) were constant. Recent reports of transient expression of DNA introduced into plant protoplasts suggests that nonintegrated DNA will express at detectable levels in plant cells (20). It is, however, possible that single or very low numbers of nonintegrated T-DNA copies per cell would not be detectable in leaf discs. Though not conclusive, our data suggest that transfer and integration are coupled phenomena that are not uncoupled by any of the avirulent mutants tested.

The weak transfer of markers by all three *virE* mutants indicates that this function is not absolutely essential. Failure to observe rare or attenuated galls from *virE* mutants might mean that there is a threshold number of transformed plant cells for gall induction that is never reached by *Agrobacterium* containing this mutation. Alternatively, the environmental/physiologic conditions may be more permissive for weak virulence in leaf discs *in vitro* than in wounded cells *in vivo*. Further analysis is necessary to determine whether the *virE* mutants transfer a normal, defined T-DNA at very low frequency or whether the low frequency might be due to abnormal T-DNA processing. The *virE* function has been reported to be complemented by coinfection with strains of *Agrobacterium* containing an intact *vir* region (21).

The results obtained from transformation in a *virC* mutant were particularly interesting. Two *virC* mutants separated by 700 nucleotides (S.S. and E.W.N., unpublished data) showed a marked preference for transformation with the *trans* pMON501 T-DNA over the *cis* A348 T-DNA. The simplest explanation for this observation is that pMON501 mimics a natural intermediate form of the T-DNA that is created by the *virC* gene product during the transfer process. Such an intermediate has been reported recently by Koukolikova-Nicola *et al.* (10). This circular intermediate, containing a single copy of the 25-base-pair border sequence, would be much smaller than the Ti plasmid. This size differential may be critical for efficient T-DNA transfer and/or integration. Thus, *virC* mutants are capable of transferring the *cis* configuration of the T-DNA (in a 250-kb plasmid) at a very low frequency, whereas they are capable of transferring the *trans* configuration of the T-DNA (in an 18-kb plasmid) at much higher frequency, but still less than wild-type frequency. The most likely explanation of the bias toward transfer of a binary vector is that the *virC* gene product is involved in generation of a T-DNA intermediate. Such a conclusion must await a more detailed analysis of these gene products. We cannot exclude the possibility that the *virC* gene product facilitates the action of an additional *vir* gene product on the Ti plasmid.

Since pMON501 is not transferred at wild-type frequency in *virC* mutants, it may not be a good mimic of a natural intermediate or the *virC* product may be important for a subsequent processing step that is not essential but increases transfer frequency. If the binary T-DNA mimics the T-DNA intermediate described by Koukolikova-Nicola *et al.* (10), a single border sequence should be sufficient to result in efficient transformation in a *virC* mutant strain. We have confirmed this by demonstrating that a binary vector with a single border can transform plant cells as efficiently as pMON501 with two borders (R.B.H. and H.J.K., unpublished data). Whether the borders in the intermediate are recognized by additional *vir* gene products during integration into plant DNA remains to be determined.

It is interesting to note that *virC* mutants are not entirely avirulent. These mutants give a weak tumorous response or altered tumor morphology on some plants but are avirulent on others (8, 9). This, combined with the present experiments, would seem to indicate that the T-DNA intermediate is not essential to the transfer process but that the smaller intermediate is far more efficiently transferred. In fact, unlike octopine- and nopaline-type Ti plasmids, a limited host range Ti plasmid, pTiAg162, lacks a functional equivalent of the *virC* gene product (M. Yanofsky, personal communication). The inability to form a T-DNA intermediate may be partly responsible for the observed limited host range.

The leaf disc transformation system has allowed us to examine a comprehensive set of virulence mutations in a much more sensitive manner than has been possible previously. The results have led to the conclusion that mutations in *virC* and *virE* do not abolish T-DNA transfer and integration. Furthermore, when the T-DNA has been excised from the Ti plasmid and placed in a small (<20 kb) plasmid vector, *virC* is not needed for efficient T-DNA transfer.

Note Added in Proof: Recently Gardner and Knauf (22) reported that *virE* mutants can transfer potato spindle tuber viroid (PSTV) to inoculated tomato plants, resulting in systemic infection, but could not cause formation of a gall. This confirms our observation that *virE* mutants are capable of DNA transfer, but we are also able to detect stable integration, perhaps because the leaf discs provide a more

sensitive assay than gall induction. They also report that a *virC* mutant does not transfer PSTV to infected plants. This discrepancy with our observations is probably due to differences in assignment of particular mutants to the *vir* region loci.

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