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The genetic and transcriptional organization of the *vir* region of the A6 Ti plasmid of *Agrobacterium tumefaciens*

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The genetic transformation of plant cells by *Agrobacterium tumefaciens* is mediated by the genes of the Ti plasmid *vir* region. To determine the genetic and transcriptional organization of the *vir* region of pTiA6, *vir* plasmid clones were saturated with insertion mutations of a Tn*3-lacZ* transposon. This element is both an insertion mutagen and a reporter for the expression of the sequences into which it has inserted. One hundred and twenty-four *vir*::Tn*3-lac* insertions were analyzed for their mutagenic effect on *Agrobacterium* virulence, and for their expression of β-galactosidase activity, the *lacZ* gene product, in vegetative bacteria and in bacteria cocultivated with plant cells. These data in conjunction with genetic complementation results show that the pTiA6 *vir* region contains six distinct *vir* complementation groups: *vir*A, *vir*B, *vir*C, *vir*D, *vir*E and *vir*G. Mutations in these loci eliminate (*vir*A, *vir*B, *vir*D and *vir*G) or significantly restrict (*vir*C and *vir*E) the ability of *Agrobacterium* to transform plant cells. Each of the *vir* loci corresponds to a single *vir* transcription unit: *vir*A is constitutively expressed and non-inducible; *vir*B, *vir*C, *vir*D and *vir*E are expressed only upon activation by plant cells; and *vir*G is both constitutively expressed and plant-inducible. The two largest *vir* operons, *vir*B and *vir*D, are probably polycistronic. The pTiA6 *vir* region also contains plant-inducible loci (*pin*) which are non-essential for virulence.

Key words: Agrobacterium vir genes/plant–bacteria interaction/Ti *vir* genetic complementation/Ti *vir* expression/Tn*3*-HoHo1 mutagenesis

Introduction

The soil phytopathogen *Agrobacterium tumefaciens* genetically transforms plant cells to cause crown gall, a neoplastic disease of dicotyledonous plants (Caplan *et al.*, 1983; Nester *et al.*, 1984; Gheysen *et al.*, 1985). During transformation the bacterium transfers a specific segment of DNA, the T-DNA, from its large (> 200 kb) Ti plasmid to the plant cell nucleus. Expression of T-DNA genes within the transformed cell results in its neoplastic growth and the synthesis of novel compounds called opines. The T-DNA transfer process is remarkable in that it carries out a complex series of events: a specific segment of DNA is recognized in and mobilized from the Ti plasmid, transferred across the cell walls of the bacterium and plant cell, and integrated as a linear non-permuted fragment into the plant nuclear genome. The molecular details of these events are not known, and research has focused on the three sets of genetic elements identified to be essential for transfer — the T-DNA border sequences, the chromosomal virulence *chv* loci and the Ti plasmid virulence *vir* loci.

During transfer the T-DNA acts solely as a structural element, since its internal portion can be fully deleted without affecting its transfer (Zambryski *et al.*, 1983; de Framond *et al.*, 1983). In the Ti plasmid the T-DNA is defined and bounded by identical 25-bp direct repeats (Wang *et al.*, 1984, and references therein). Because only DNA between these T-DNA borders is seen to be transferred to the plant genome, these sequences are likely to be the structural substrates of the proteins that directly mediate the transfer process. While the T-DNA borders are *cis*-acting sequences, the *chv* and *vir* loci function *in trans* (Garfinkel and Nester, 1980). The *chv*A and *chv*B loci are essential for virulence, specify the binding of *Agrobacterium* to plant cells and are constitutively expressed in the bacterium (Douglas *et al.*, 1985). In contrast, the expression of the *vir* loci is tightly regulated. The *vir* loci are located within the Ti plasmid *vir* region, and the expression of *vir* region sequences becomes induced to high levels when *Agrobacteria* are cocultivated with actively growing plant cells (Stachel *et al.*, 1985a, 1986). This induction is mediated by small phenolic compounds produced in the exudates of these cells (Stachel *et al.*, 1985b), and is accompanied by specific T-DNA-associated molecular events (Koukolíková-Nicola *et al.*, 1985; Stachel *et al.*, 1985b, 1986). Thus the T-DNA transfer process is active in nature only when *Agrobacterium* is in the presence of plant cells, and the products of the *vir* region genes direct the events of this process.

While the importance of the *vir* region as the mediator of T-DNA transfer is clear, an understanding of the genetic and transcriptional organization of this region is incomplete. To date, mutational analyses have indicated that a continuous region of ~40 kb is essential for virulence (Garfinkel and Nester, 1980; Holsters *et al.*, 1980; Ooms *et al.*, 1980). While several studies on the genetic organization of this region in a number of different Ti plasmids have been reported (for example, Klee *et al.*, 1983; Hille *et al.*, 1984; Knauf *et al.*, 1984; Lundquist *et al.*, 1984), this region has not been fully saturated by mutations, and the organization of the existing *vir* mutations into distinct *vir* complementation groups is not well established. Preliminary *vir* expression studies have suggested that certain *vir* region sequences are constitutively transcribed (Gelvin *et al.*, 1981) while other sequences are plant-activated (Stachel *et al.*, 1986); however, these studies do not provide a comprehensive view of the transcription of the *vir* region during vegetative growth or cocultivation with plant cells. Here we characterize in detail the genetic and transcriptional organization of the *vir* loci of the pTiA6 octopine-type Ti plasmid.

Results

Experimental approach

To analyze the genetic and transcriptional organization of the pTiA6 *vir* region, the experimental strategy of Figure 1 was followed. This strategy is based on a set of *vir*::*lac* insertion molecules generated by inserting a promoterless *lacZ* gene throughout plasmid clones of pTiA6 *vir* region sequences. Since each *vir*::*lacZ* insertion is both a potential *vir* mutation and a *vir*::*lacZ* fusion gene, the insertion set allows the determination of (i) the

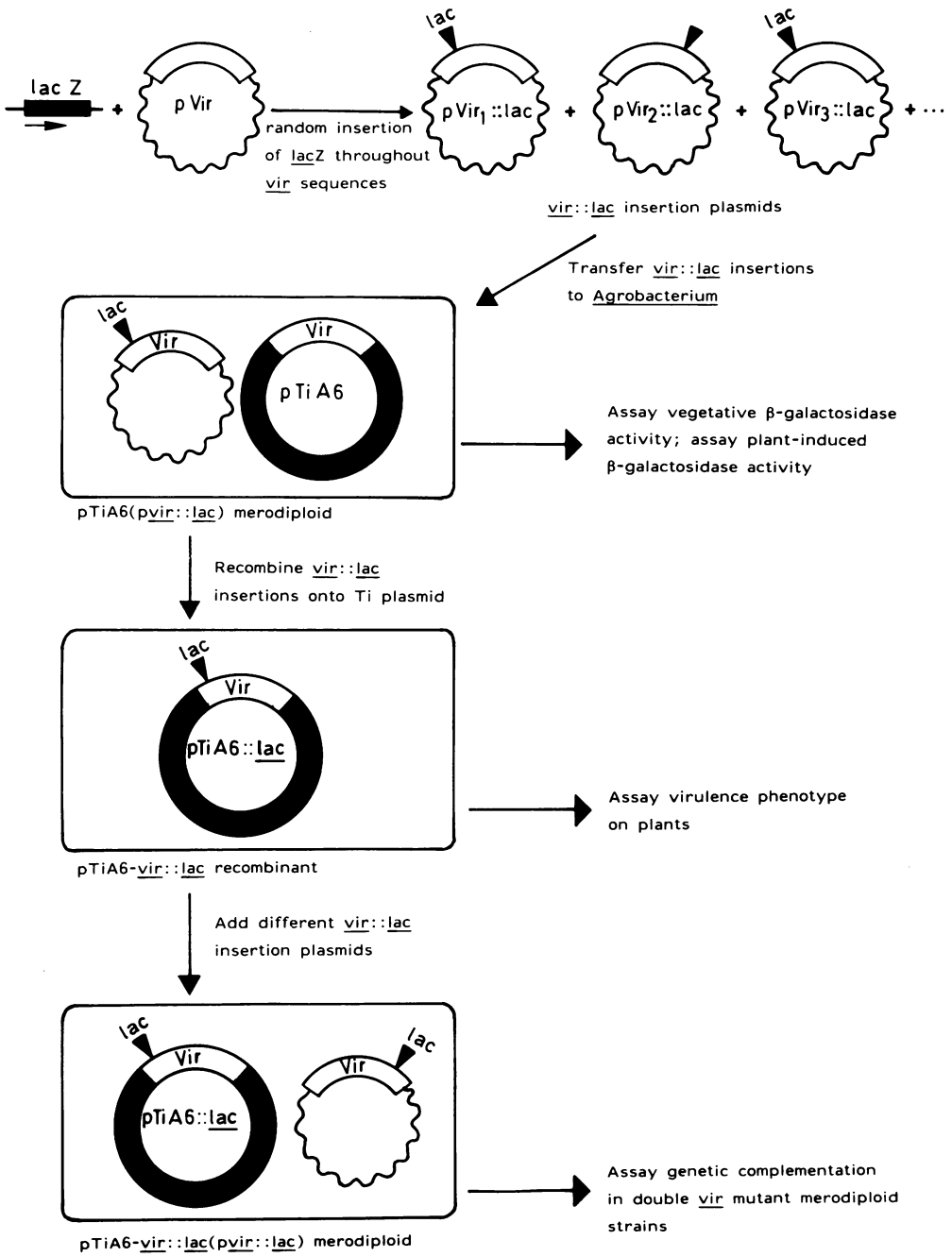

Fig. 1. Experimental strategy for the analysis of the genetic and transcriptional organization of the *vir* region of the A6 Ti plasmid of *Agrobacterium*.

vir region sequences required for virulence, and their genetic organization; and (ii) the *vir* region sequences which are transcribed, their transcriptional orientation and the conditions under which they are expressed.

To assess the mutagenic effect of an insertion, it is introduced onto pTiA6 by homologous recombination in *Agrobacterium* and the resulting recombinant is tested for its ability to cause tumors on plants. *Vir* insertion mutations are subsequently organized into separate *vir* loci in genetic complementation experiments using *vir* merodiploid strains. Each merodiploid contains a pTiA6 *vir* mutant and a complementing plasmid that carries a *vir*::*lac* insertion or a *vir* deletion; if wild-type virulence is restored in a merodiploid, the two *vir* mutations that it carries are within separate *vir* loci. The structural limits of each *vir* locus are determined from the positions of flanking insertions that are either virulent or within adjacent *vir* loci.

The promoterless *lacZ* element used as the insertion mutagen is also a reporter for the transcription of the sequences into which it has inserted. Expression at the insertion site is monitored by assaying for β-galactosidase activity, the *lacZ* gene product. Because *Agrobacterium* does not have its own β-galactosidase activity (Plessis *et al.*, 1985; Stachel *et al.*, 1985a), significant activity in a cell that carries a *vir*::*lac* insertion indicates that the *lacZ* sequences are located downstream of, and in the same transcriptional orientation as, a *vir* region promoter undergoing active transcription. Such expressed insertions are designated '*vir*::*lac* fusion genes'.

The expression of *vir* region sequences becomes activated when

Fig. 2. Genetic and transcriptional organization of the *vir* region of pTiA6. The respective map positions, defined genetic boundaries and transcriptional orientation of the *vir* region loci *pin*F, *vir*A, *vir*B, *vir*G, *vir*C, *vir*D and *vir*E are indicated by arrows above the *Sal*I, *Eco*RI and *Bam*HI restriction enzyme maps of the pTiA6 *vir* region. pVCK219, pVCK221 and pVCK225, three cosmid clones that contain pTiA6 *vir* region sequences, are shown below as horizontal lines. These clones were mutagenized with the Tn*3*-*lacZ* transposon Tn*3*-HoHo1, to generate 124 independent pSM(*vir*::*lacZ*) insertion plasmids. The figure shows four distinct properties for each Tn*3*-HoHo1 insertion: its map position, the orientation of its *lac* sequences, the β-galactosidase expression phenotype of its corresponding pTiA6(pSM*vir*::*lac*) merodiploid strain and the virulence phenotype of its corresponding pTiA6 recombinant strain. Each insertion has been assigned an identifying number and its map position is indicated by the vertical line adjacent to its number. Those insertions whose Tn*3*-HoHo1 *lac* sequences are oriented, with respect to the pTiA6 *vir* map, left-to-right (LR) are drawn above, and those oriented right-to-left (RL) are drawn below, the horizontal lines of the cosmid clones respectively. The length of the vertical line that represents an insertion indicates whether its pTiA6(pSM*vir*::*lac*) strain exhibits plant-inducible β-galactosidase activity (>4-fold change from its vegetative activity): insertions represented by short lines are non-inducible, and by long lines are plant-inducible. The vertical arrows indicate insertions that give high levels (>25 U) of vegetative β-galactosidase activity. The asterisk above insertion 368 indicates that this insertion is non-inducible when located on its insertion plasmid (pSM368) and becomes inducible when recombined onto pTiA6(pTi368). The *vir*::*lac* insertions were recombined onto pTiA6 to assess their effect on virulence. Insertions marked with open circles are fully virulent, with closed circles are avirulent and with closed triangles are partially virulent on *K. daigremontiana* leaf wounds. Note that *vir*C and *vir*E mutants show full and attenuated virulence respectively on *N. tabacum* leaf discs. Unmarked insertions were not tested for virulent phenotype. The separate *vir* complementation groups are defined by the complementation analysis presented in Table I and Figure 3. Note that insertions 34, 35, 37, 38 and 41 are carried by pVCK242; pVCK242 is a pTiA6 *vir* clone that is identical to pVCK221 except that it also has *Sal*I fragment 34a at its left end and *Sal*I fragment 30 at its right end (Knauf and Nester, 1982).

Agrobacterium is cocultivated with actively growing plant cells (Stachel *et al.*, 1985a, 1986). It is also likely that certain *vir* region sequences are constitutively expressed in both vegetatively grown and cocultivated bacteria. Thus the level of β-galactosidase activity expressed by each insertion of the insertion set is determined under two different conditions, vegetative growth and cocultivation with plant cells. Insertions effecting significant levels of vegetative β-galactosidase activity are designated 'constitutive insertions' and identify constitutively expressed sequences. Insertions that express β-galactosidase activity altered by cocultivation with plant cells are designated 'plant-inducible insertions' and identify plant-inducible sequences.

Construction of vir::lac insertions

The *vir*::*lac* insertions were generated in *Escherichia coli* by Tn*3*-HoHo1 transposon mutagenesis (Stachel *et al.*, 1985a) of three *vir* cosmid clones, pVCK219, pVCK221 and pVCK225 (Knauf and Nester, 1982; Figure 2), which contain overlapping portions of the pTiA6 *vir* region and replicate in both *E. coli* and *Agrobacterium*. Tn*3*-HoHo1 is a Tn*3*-*lac* transposon that carries a promoterless *lacZ* gene that is in-frame through the left-end of the element. Tn*3*-HoHo1 generates random insertional mutations in, and *lac* fusions of, genes within the target sequences. Both transcriptional and translational *lacZ* fusions are obtained with Tn*3*-HoHo1: the transcriptional fusions express a β-galactosidase protein completely encoded within Tn*3*-HoHo1 that is dependent on external transcription, while the translational fusions express chimeric β-galactosidase fusion proteins that depend both on external transcription and translation. Statistically, one out of three gene fusions will be translational.

One hundred and twenty-four independent insertion plasmids were chosen as a representative set of *vir*::Tn*3*-HoHo1 insertions. An attempt was made to have insertions of both orientations of Tn*3*-HoHo1 (with regard to its *lacZ* sequence) every 0.5 kb across the pTiA6 *vir* region, from *Sal*I fragment 4 to *Sal*I fragment 21. The set of insertion plasmids contains 37 pVCK219, 31 pVCK221 and 56 pVCK225 derivatives (Figure 2). In the following presentation, insertions are used in two different molecular environments: (i) within their original insertion plasmids; and (ii) within their pTiA6 recombinants. These molecules are respectively indicated by the identifying number of the insertion preceded by the prefixes pSM and pTi.

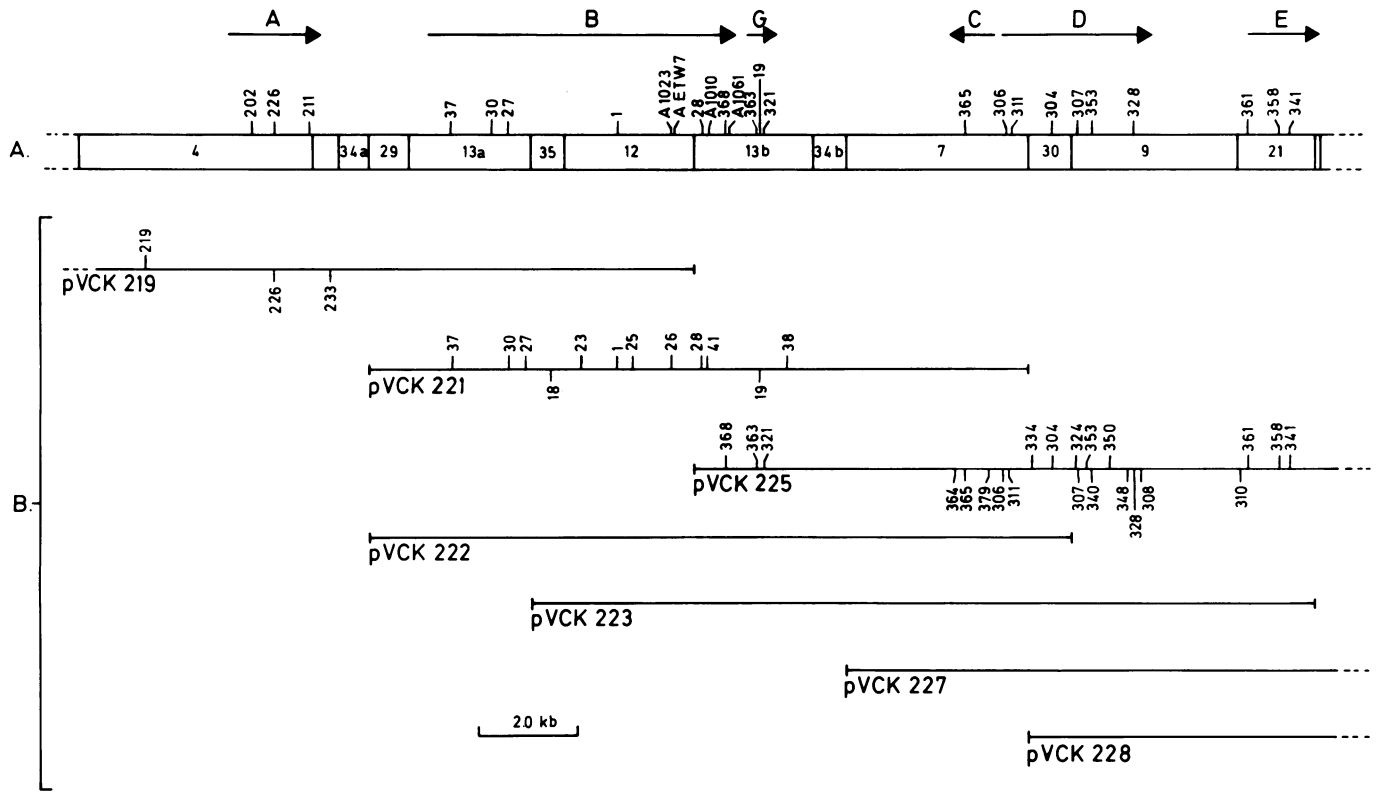

Fig. 3. pTiA6 *vir* mutants and pVir cosmids used in the *vir* genetic complementation experiments of Table I. **(A)** The map positions and identifying numbers of the mutations of the tested pTiA6 *vir* mutants are indicated above the *Sal*I restriction map of the *vir* region. **(B)** pVir cosmids used to complement the pTiA6 *vir* mutants. Several of these cosmids are pSM(*vir*::*lac*) insertion plasmids and carry Tn*3*-HoHo1 insertions. The position of an insertion is given by the vertical line next to its identifying number. Those insertions whose Tn*3*-HoHo1 *lac* sequences are oriented left-to-right are indicated above, and those of the opposite orientation are indicated below, the horizontal lines that represent the pTiA6 *vir* sequences carried by the parental pVCK cosmid clones.

Insertion mutagenesis of the pTiA6 vir region

The 124 insertion plasmids were transferred by conjugation to *Agrobacterium* strain A348, which contains wild-type pTiA6, to give a set of 124 A348(pSM*vir*::*lacZ*) merodiploid strains. Throughout the rest of the present study these strains are referred to as pTiA6(pSM*vir*::*lac*) strains, to indicate that they carry pTiA6 and a *vir*::*lac* insertion plasmid. In 112 of the pTiA6-(pSM*vir*::*lac*) strains the *vir*::Tn*3*-HoHo1 insertion was introduced onto pTiA6 by double homologous recombination, and two independent pTiA6::Tn*3*-HoHo1 recombinants per insertion were isolated. Each recombinant strain was inoculated onto leaf wounds of *Kalanchoe daigremontiana* to assess its virulence.

The pTiA6::Tn*3*-HoHo1 recombinants display three general phenotypes for tumor formation, wild-type virulence, avirulence and attenuated virulence (Figure 2). Forty-eight recombinants are fully virulent and 60 are avirulent. The avirulent insertions fall into four discrete regions that are separated by several virulent insertions. Two of these regions are bisected by single virulent insertions that are closely flanked by avirulent insertions: insertion 41 in *Sal*I fragment 13b and insertion 324 in *Sal*I fragment 9. The virulent phenotype of two segments of ~1.0 kb, between insertions 231 and 219 in *Sal*I fragment 4 and between insertions 368 and 363 in *Sal*I fragment 13b, were not assessed by insertional mutagenesis.

Four insertions give attenuated virulence; pTi364, pTi365, pTi379 and pTi361 induce tumors that begin to appear only 30 days post-inoculation. After 60 days, tumors of wild-type size are seen; however, the tumor tissue does not occur throughout the wound site but is limited to one or two sites that, from several test inoculations, are randomly distributed. The 364, 365 and 379 insertions are located within a 1.2-kb region of *Sal*I fragment 7. Insertion 361 is located within *Sal*I fragment 21; more rightward *Sal*I fragment 21 mutations are avirulent on *Kalanchoe*.

Several of the avirulent and attenuated virulent recombinants were also inoculated on *Nicotiana tabacum* leaf discs and *Nicotiana glauca* stem wounds. The tumor-inducing phenotype of each tested recombinant on these two assay systems is identical to that observed on *Kalanchoe* with the exception that the pTi364, pTi365 and pTi379 strains are fully virulent on *N. glauca* stem wounds, and all the *Sal*I fragment 21 mutations, scored as avirulent on *Kalanchoe*, give attenuated virulence on *N. tabacum* leaf discs. These results suggest that these two groups of insertion mutations are within *vir* loci which are not absolutely essential for transformation of all plant species or tissues.

Genetic organization of the pTiA6 vir region

The above analysis identifies the areas of the pTiA6 *vir* region that encode virulence functions. The complementation analysis presented in Figure 3 and Table I demonstrates that these areas are organized into six distinct *vir* complementation groups, *vir*A, -B, -C, -D, -E and -G. An important aspect of this analysis is that Tn*3*-HoHo1 is both a direct and a polar mutagen; insertion of Tn*3*-HoHo1 into a locus directly disrupts coding sequences and, when within a polycistronic unit, disrupts the regulation of expression of downstream cistrons. Thus the mutant phenotype of each Tn*3*-HoHo1-generated pTiA6 *vir* mutant should only be complemented by plasmids that carry a complete and intact copy of the mutant *vir* locus. While this rule is generally valid, exceptions can occur, as presented below.

virA. Results obtained with the avirulent mutants pTi202, pTi226

Table I. Genetic complementation of pTiA6 *vir* mutants

Merodiploid mutant pTiA6/pVir cosmid		Virulent phenotype	Merodiploid mutant pTiA6/pVir cosmid		Virulent phenotype
*vir*A	202 pSM219	+	*vir*D	306 pSM306	−
	226 pSM226	−		353 pSM334	−
	211 pSM233	+		pSM340	−
				pSM350	−
*vir*B	A1023 pSM19	+		pSM348	−
	AETW7 pSM23	−		pSM308	−
	pSM25	−		pSM358	+
	pSM26	−			
	pSM28	−		307 pSM365	+
	pSM41	+		311 pSM311	−
				328 pSM324	+
	37 pVCK222	+		pSM307	−
	27 pVCK223	−		pSM328	−
	1 pSM37	−			
	28 pSM27	−		328 pVCK227	+
	pSM26	−		pVCK228	−
	pSM28	−			
				311 pVCK222	−
	38 pVCK222	+		pVCK223	+
	A1010 pVCK223	−			
	A1061 pSM18	−		304 pVCK222	+
	pSM19	+		pVCK223	+
				pVCK227	+
	30 pVCK219	−		pVCK228	−
	37 pSM41	+		pSM365	+
				pSM311	−
	368 pVCK222	+		pSM324	+
	pVCK223	−		pSM307	+
	pSM28	−		pSM328	+
	pSM363	−			
				306 pSM304	−
*vir*G	363 pVCK222	+		311	
	19 pVCK223	+		304	
	321 pSM28	+			
	pSM19	−		307 pSM304	+
	pSM304	+		353	
	pSM321	−		328	
	pSM368	+			
				358 pSM311	+
*vir*C	365 pSM364	±		pSM304	+
	pSM311	+		pSM307	+
	pSM304	+		pSM328	+
	pSM307	+			
	pSM324	+	*vir*E	358 pSM361	±
	pSM328	+		341 pSM358	−
	pSM19	+		pSM341	−
	pSM379	±			
	pSM365	±		361 pSM310	+
				pSM361	±
				pSM358	±
				pSM341	±
				358 pVCK223	−
				pVCK225	+

One hundred and fifty-seven independent merodiploid strains were constructed and assayed for their ability to induce tumors on *K. daigremontiana* leaf wounds. Each strain contains two plasmids, a pTiA6 derivative that is mutant for a *vir* locus (mutant pTiA6), and a complementing cosmid that carries a portion of the pTiA6 *vir* region (pVir cosmid). The pTiA6 *vir* mutants and pVir cosmids used are diagrammed in Figure 3. The experimental results are presented in sets of 2 × 2 matrices, and indicate that each pTiA6 mutant (noted to the left) in the set has been tested with each pVir cosmid (noted to the right). For example, for *vir*A, three pTiA6 *vir*A mutants were tested with three pVir cosmids and thus nine independent merodiploid strains were constructed and assayed: pTi202(pSM219), pTi226(pSM219) and pTi211(pSM219) are virulent; pTi202(pSM226), pTi226(pSM226) and pTi211(pSM226) are avirulent; and pTi202(pSM233), pTi226(pSM233) and pTi211(pSM233) are virulent. −, avirulent; +, virulent; ±, partially virulent.

Table II. Dependence of the induced expression of 3′ *vir*B insertions on 5′ *vir*B sequences

Strain	β-Galactosidase units		
	− induction	+ induction	Δ units
pTiA6/pSM243	4.14	291	70.3
pTi243	1.92	84.7	44.1
pTiA6/pSM368	6.01	9.72	1.62
pTi368	3.27	50.3	15.4
pTiA6/pSM1	7.20	589	81.8
pTi1	2.51	212	84.5
pTiA6/pSM1cd	4.98	6.52	1.31
pTi1cd	2.06	176	85.4
pTiA6/pSM26	5.32	89.6	16.8
pTi26	1.64	41.2	25.1
pTiA6/pSM26cd	3.40	3.02	1.26
pTi26cd	1.71	35.9	21.0

The vegetative (− induction) and plant-induced (+ induction) β-galactosidase activities of different pTiA6(pSM*vir*::*lac*) merodiploid strains and their corresponding pT(*ivir*::*lac*) recombinant strains were measured, and the inducibility (Δ units) of each strain was calculated. The insertion plasmids used to construct these strains are given in Figure 2 and in the text. Note that both the basal and induced units affected by an insertion are ~3- to 4-fold greater when it is located on its insertion plasmid than when on its pTiA6 recombinant. This probably reflects the higher copy number of the insertion plasmid than the Ti plasmid in *Agrobacterium*. Also note that while insertion 368 is not inducible on pVCK225, the closely adjacent *vir*G insertions 363 and 321 are (Table III); thus, *vir*G expression is independent of upstream *vir*B expression.

and pTi211, which carry insertions within a 1.8-kb region of *Sal*I fragment 4, define the *vir*A locus. These mutants are complemented to wild-type virulence by pSM219 and pSM233, which carry insertions outside the 1.8-kb *vir*A region, but not by pSM226, which carries an insertion within *vir*A. The positions of the wild-type insertions 203 and 201 (Figure 2), show *vir*A has a maximum size of 2.5 kb.

virB. The *vir*B locus is defined by complementation results obtained for 10 avirulent mutations (Figure 3) that span an 8.5-kb region from *Sal*I fragment 13a through to the left end of *Sal*I fragment 13b. Only plasmids that carry a complete and intact copy of this region (with the exception of pSM41, see below) complement these mutants.

The *vir*B region has been reported to contain six separate complementation groups (Iyer *et al.*, 1982), one of which cannot be *trans*-complemented (Klee *et al.*, 1982; Iyer *et al.*, 1982). Here, this region behaves as a single locus, and expression results presented in Table II further support this conclusion. Also, all *vir*B mutants are observed to be *trans*-complementable. The maximum left end of *vir*B is defined by the position of the wild-type insertion 204 (Figure 2). The maximum right end of *vir*B is given by insertion 363 which is within *vir*G, and from the left end of *vir*G, defined by DNA sequence analysis (Winans *et al.*, submitted). *Vir*B has a maximum size of 9.5 kb.

Insertion 41, located within *Sal*I fragment 13b (0.1 kb to the right of the *vir*B insertion mutation 28), behaves differently from other *vir*B insertions. pTi41 is fully virulent and pSM41 complements *vir*B mutants, suggesting that insertion 41 is located outside and to the right of *vir*B. However, three pTiA6 mutants, A1010, pTi368 and A1061 (Figure 3), which carry transposon mutations located ~0.1, 0.6 and 0.7 kb to the right of insertion 41 respectively, complement as *vir*B mutants (Table I). Thus insertion 41 must be within *vir*B sequences that do not encode a *vir* function, such as an untranslated intercistronic region. Also,

Table III. Vegetative and plant-induced β-galactosidase activities of representative pTiA6(pSM*vir*::*lac*) strains

Locus/ orientation	Strain pTiA6(pSM*vir*::*lac*)	β-Galactosidase units − induction	β-Galactosidase units + induction	Δ units
A/LR	202	48.4	55.3	1.13
	211	28.2	25.5	0.90
A/RL	240	3.10	2.45	0.79
	210	3.05	5.16	1.72
B/LR	243	3.24	283	87.3
	37	3.60	220	61.1
	30	9.53	548	57.5
	16	5.60	127	22.7
	1	7.20	589	81.8
	11	6.28	310	49.4
	26	5.32	89.6	16.8
	41	9.20	85.4	9.28
B/RL	217	3.18	2.75	0.87
	18	3.99	2.58	0.65
	40	3.53	3.43	0.97
	32	3.94	2.46	0.62
G/LR	363	64.8	879	13.6
	321	77.0	981	12.7
G/RL	19	3.68	3.26	0.89
C/LR	364	5.66	5.22	0.92
C/RL	379	3.42	61.5	17.9
	365	4.55	69.7	15.3
D/LR	335	3.55	70.8	19.9
	352	3.29	73.8	22.4
	304	5.91	624	106
	324	3.50	199	56.9
	353	3.49	59.6	17.1
	350	9.70	74.9	7.6
	370	3.87	36.30	9.38
D/RL	306	2.85	3.68	1.29
	307	3.12	2.78	0.89
	328	2.86	2.07	0.72
E/LR	333	3.59	45.4	12.6
	358	59.9	2941	49.1
	341	2.07	205	29.6
E/RL	362	2.48	1.9	0.77
	330	4.82	5.07	1.05
*pin*F/LR	231	6.14	174	28.3
	219	10.3	826	80.2
	208	4.24	69.4	16.4
*pin*F/RL	213	2.81	2.23	0.79
	222	2.63	2.55	0.97
3'G/LR	38	26.1	156	5.98
	326	28.1	127	4.52

LR, the orientation of the *lac* sequences of the insertion is leftward to rightward with regard to the pTiA6 *vir* region map of Figure 2. RL, the orientation of the *lac* sequences of the insertion are rightward to leftward. Δ units, the ratio of plant-induced activity:vegetative activity.

since this insertion is closely flanked by avirulent mutations, it must be functionally non-polar for downstream *vir*B transcription.

A possible explanation for the non-polarity of insertion 41 is that in certain instances Tn*3*-HoHo1 promotes the expression of sequences downstream of its insertion site. If this occurs for an insertion located within an intercistronic region of an operon, the insertion will be non-polar for downstream cistrons and display a wild-type phenotype. Since the *bla* gene of Tn*3*-HoHo1 is located adjacent to its right inverted-repeat (Stachel *et al.*, 1985a), inefficient termination of *bla* transcription of an insertion could result in the transcription of downstream sequences.

virG. Three avirulent mutants, pTi363, pTi19 and pTi321, carry insertion mutations within a 0.2-kb region of *Sal*I fragment 13b. These mutations do not cross-complement and define the *vir*G locus. *Vir*G and *vir*B are genetically and phenotypically distinct: pVCK222, pVCK223 and Tn*3*-HoHo1 derivatives of pVCK225, which lacks the left 8 kb of *vir*B, restore virulence to *vir*G but not to *vir*B mutants; *vir*G and *vir*B mutants cross-complement; and plant-induced *vir* expression occurs in *vir*B but not *vir*G mutant bacteria (Stachel and Zambryski, 1986). *Vir*G has a maximum size of 1.2 kb. Its left end has been defined by DNA sequence analysis (Winan *et al.*, submitted) and the maximum extent of its right end by the position of wild-type insertion 38 (Figure 2).

virC. The insertions 364, 365 and 379 do not cross-complement, and define the *vir*C locus. *Vir*C and *vir*D are distinct on criteria of phenotype, complementation and transcriptional orientation: while *vir*C mutants display an attenuated virulent phenotype, *vir*D mutants are avirulent; the *vir*C mutant pTi365 is complemented by several *vir*D insertion cosmids, and several *vir*D mutants are complemented by pSM365; and *vir*C and *vir*D are divergently transcribed (Table III and Figure 2). The positions of virulent insertion 337 and of *vir*D insertion 306 respectively mark the maximum left and right boundaries of *vir*C (Figure 2). *Vir*C has a maximum size of 2.0 kb.

virD. The *vir*D locus is defined by complementation results obtained for insertion mutations located within a 4.5-kb region that extends rightward from the right end of *Sal*I fragment 7, through *Sal*I fragment 30 and into the left end of *Sal*I fragment 9. With the exceptions of insertions 304 and 324 (see below), *vir*D mutations do not cross-complement. Furthermore, complementation of several insertions within the 4.5-kb *vir*D region is not obtained if the complementing *vir* cosmid carries only a portion of this region. The position of the virulent pTi359 insertion marks the maximum right end of *vir*D. The maximal left end is defined by the position of the *vir*C insertion 379.

While insertion 324 is in the middle of *vir*D, pTi324 is fully virulent and *vir*D mutants are complemented by pSM324. Thus insertion 324 is analogous to the virulent *vir*B insertion 41 and potentially identifies the position of a *vir*D intercistronic sequence. Further anomalous results are obtained with the avirulent insertion 304, which is located within *Sal*I fragment 30 and bounded to its left by avirulent insertion 352, and to its right by virulent insertion 324 and avirulent insertion 307. Unlike other *vir*D mutants, pTi304 is complemented by plasmids which do not carry an intact *vir*D locus but only the left portion of *vir*D (*Sal*I fragments 7 and 30). Furthermore, pSM304 restores virulence to *vir*D mutants that map rightward, but not leftward, of the site of the 304 insertion. These results may be interpreted to indicate that *vir*D is two distinct complementation groups that fall to the left and right of the site of insertion 324. However, this interpretation conflicts with the extensive complementation results obtained with other *vir*D mutations (Table I). A more likely explanation for insertion 304 is that while it disrupts a leftward *vir*D cistron, it still allows the functional expression of *vir*D cistrons that fall rightward of its site of insertion. This expression may be promoted by the Tn*3*-HoHo1 *bla* gene, as discussed above for the wild-type *vir*B insertion 41.

virE. Insertions 361, 358 and 341, which are located within a 1.2-kb region of *Sal*I fragment 21 and do not cross-complement,

define the *vir*E locus. Virulent insertion 362 (Figure 2), which maps ~300 bp rightward of the left end of *Sal*I fragment 21, marks the left end of *vir*E. pVCK223 (Figure 3), which carries *vir* sequences inclusive and leftward of *Sal*I fragment 21, does not complement pTi358; thus the right end of *vir*E extends beyond this fragment. Since Tn*3*-HoHo1 insertions located just within the right end of *Sal*I fragment 21 are avirulent on *Kalanchoe* and complement as *vir*E mutations, and insertions located just within the left end of *Sal*I fragment 18 are fully virulent (P.Totten and S.E.Stachel, unpublished data), the right terminus of *vir*E probably occurs within the ~150-bp *Sal*I fragment located between *Sal*I fragments 21 and 18. *Vir*E has a maximum size of 2.0 kb.

As described above, *vir*E mutants are avirulent on *Kalanchoe* but give attenuated virulence on *N. tabacum* leaf discs. Thus *vir*E, like *vir*C, is not absolutely essential for *Agrobacterium* transformation of all plant species. We note that another *vir* locus, designated *vir*F, has been reported to map ~7 kb rightward of *vir*E (Hooykaas *et al.*, 1984); extensive Tn*3*-HoHo1 mutagenesis of this region of pTiA6 indicates it has no significant role in virulence on *Kalanchoe*, tobacco or tomato (P.Totten and S.E. Stachel, unpublished data).

Constitutive and plant-induced vir expression

The set of 124 pTiA6(pSM*vir*::*lac*) merodiploid strains was used to identify constitutively expressed and plant-inducible *vir*::*lac* insertions (Figure 2). The vegetative and plant-induced β-galactosidase activities of each strain were determined, and representative numerical data are presented in Table III. As described below, these data identify (i) the *vir* loci which are constitutively expressed; (ii) the *vir* loci which are plant-inducible, including loci not required for *Agrobacterium* pathogenicity; and (iii) the respective transcriptional orientation of each expressed locus. These results, in total, generate a transcription map of the pTiA6 *vir* region (Figure 2).

Constitutively expressed vir loci. Vegetative β-galactosidase activity was measured following growth in minimal medium. Of the 124 insertions tested, 117 give <10 units of uninduced activity, indicating that much of the pTiA6 *vir* region is not expressed, or is weakly expressed, during vegetative growth. In contrast, some *vir* sequences appear to be transcribed at significant levels in vegetative *Agrobacteria* since seven insertions give >25 units of uninduced activity. These insertions are indicated in Figure 2 by vertical arrows and include the *vir*A::*lac* insertions 202 and 211, the *vir*G::*lac* insertions 363 and 321, the virulent insertions 326 and 38, which are located just rightward of *vir*G, and the *vir*E::*lac* insertion 358. Since the *vir*::*lac*Z gene of each of these constitutive insertions is oriented left to right (LR) with respect to the *vir* region, *vir*A, *vir*G and potentially *vir*E are transcribed in a left to right direction during vegetative growth. Note that of five *vir*E::*lac* LR insertions tested, only insertion 358 gives a high level of vegetative activity and thus *vir*E is probably not vegetatively expressed. In a previous analysis of pTiA6-encoded RNA, three areas of pTiA6 were shown to be transcribed at detectable levels in vegetative bacteria (Gelvin *et al.*, 1981). These areas were identified as the T-DNA region, and two segments of the *vir* region which correspond to the map positions of *vir*A and *vir*G.

Plant-inducible vir loci. Plant-induced β-galactosidase activity was measured following 16 h cocultivation of bacteria with *N. tabacum* suspension culture cells. For each insertion the ratio of its plant-induced:vegetative activity was determined and an insertion is designated plant-inducible or plant-repressible if this ratio is >4 or <0.25 respectively. Forty-eight of the 124 insertions are plant-inducible, demonstrating that the expression of much of the *vir* region becomes activated when *Agrobacterium* is exposed to plant cells. The β-galactosidase activity displayed by each of the remaining insertions is not affected by cocultivation. Because no insertions are plant-repressible, the *vir* region does not appear to contain sequences whose expression is repressed by cocultivation.

Figure 2 shows that the inducible insertions are organized into six distinct regions that respectively correspond to the positions of *vir*B, *vir*G, *vir*C, *vir*D, *vir*E and the virulent plant-inducible locus *pin*F (discussed below). Except for *vir*C, all inducible insertions are oriented right-to-left, indicating that *vir*B, *vir*G, *vir*D, *vir*E and *pin*F are expressed in this direction. The inducible *vir*C insertions 379 and 365 are oriented right-to-left and thus *vir*C is transcribed in the opposite direction to the other *vir* loci. Each inducible locus probably encodes a single transcript. For example, all insertions located within each inducible locus and oriented in the direction of inducible transcription are plant-inducible (with the exception of the *vir*E insertion 361 and the *vir*B insertions 377 and 368, discussed below); furthermore, all oppositely oriented insertions are non-inducible.

Insertion 361 is located at the 5′ end of *vir*E. Unlike other *vir*E LR insertions, this insertion is non-inducible and produces an attenuated virulent phenotype on *Kalanchoe*, and thus might be located outside the transcribed region of *vir*E and within, or closely adjacent to, its promoter. The *vir*B LR insertions 377 and 368 are non-inducible when carried on pVCK225. This plasmid lacks the proximal region of *vir*B and thus the *vir*B promoter (see also Table II).

There are at least two distinct classes of non-inducible insertions, those within an inducible locus but oriented opposite to its direction of transcription and those within a region in which insertions of either orientation are non-inducible. This second class identifies non-inducible sequences and includes the *vir*A locus. Thus *vir*A is non-inducible and, as shown in the previous section, constitutively expressed in a left to right direction.

Interestingly, different inducible insertions exhibit different levels of induced β-galactosidase activity as well as different levels of relative inducibility (Δ units, Table III). While these results suggest that particular *vir* loci might be inducibly expressed at higher levels and/or are more inducible than other *vir* loci, several variables including transcriptional and translational efficiency as well as transcript and protein stability might also be involved. Keeping this proviso in mind, the following observations are made. Of the 48 inducible insertions, the *vir*C insertions generally give the lowest levels of induced activity while the *vir*E insertion 358 gives the highest, to suggest that *vir*C and *vir*E are the lowest and highest expressed inducible *vir* loci respectively. Also, different insertions within a single locus can give different levels of inducibility, and typically insertions located 5′ are observed to be more inducible than those 3′. For instance, of the several LR insertions of *vir*B and *vir*D, those distally located are the least inducible. Thus, proximal plant-activated transcription in these loci might be greater than distal transcription.

The most highly inducible insertions encode chimeric *vir*::β-galactosidase fusion proteins; for example the highly inducible insertions 219 (*pin*F), 304 (*vir*D) and 358 (*vir*E) were shown in a preliminary report (Stachel *et al.*, 1985a) to encode fusion proteins of ~185, 193 and 156 kd respectively. The size of each of these proteins can be used to identify the position of the translational start site of the cistron in which the insertion element

is located, i.e. since the *lac*Z gene of Tn*3*-HoHo1 encodes a β-galactosidase protein of ~148 kd (Stachel *et al.*, 1985a), the *vir*::β-galactosidase fusion proteins encoded from insertions 219, 304 and 358 are initiated ~37 (1.0 kb), 45 (1.2 kb) and 8 kd (250 bp) 5′ (leftward) respectively of the positions of their respective Tn*3*-HoHo1 insertions. These data define the potential 5′ end of *pin*F and indicate that insertion 304, which maps 1.6 kb downstream of the 5′ end of *vir*D, is within a large *vir*D cistron that begins 400 bp downstream of the start of *vir*D, and that insertion 358 is within a *vir*E cistron whose initiation site closely corresponds to the 5′ map position of *vir*E (Figure 2).

Plant-inducible sequences not required for virulence. Several insertions which do not affect virulence also give plant-inducible β-galactosidase activity. These insertions fall into two distinct classes, insertions located just 3′ of plant-inducible loci and insertions located within the plant-inducible locus *pin*F. For example, virulent insertions 326, 38, 373 and 375, which fall across a 1-kb region 3′ of *vir*G, give 4.5-, 6.0-, 7.9- and 7.4-fold plant-inducible activity respectively; and virulent insertion 359, which lies 3′ of *vir*D, gives 12-fold plant-inducible activity.

Five LR insertions located across a 2.2-kb region of the left end of the *Sal*I fragment 4 give significant levels of plant-inducible β-galactosidase activity (Table III). Because the corresponding pTiA6 recombinants of these insertions are fully virulent, they define the plant-inducible locus, designated *pin*F, whose product has no detectable role in *Agrobacterium* pathogenicity. *pin*F has a maximum size of 3.5 kb, and its maximum boundaries are marked by the non-inducible LR insertions 230 and 206. Because *pin*F expression is induced by specific plant phenolic compounds (Stachel *et al.*, 1985b), its gene product(s) may function during the bacterial−plant interaction. We note that another plant-inducible pTiA6 locus, not required for virulence, maps ~10 kb rightward of *vir*E (P.Totten and S.E.Stachel, unpublished observations). Potentially, *Agrobacterium* carries several different *pin* loci.

virB is a single transcription unit. Since *vir*B complements as a single locus and is transcribed in a left-to-right direction, it might encode a single large transcript initiated from a plant-activated promoter located within *Sal*I fragment 13a. Table II shows that *vir*B LR insertions are inducible only if linked to the 5′- terminal sequences of *vir*B. *Vir*B LR insertions carried either on pVCK219, which lacks the 3′ sequences of *vir*B, or on pTiA6, are plant-inducible. Also, the strains pTiA6 (pSM243) and pTi243 (Figure 2) express approximately equivalent levels of induction. In contrast, the *vir*B insertion 368 is inducible when located on pTiA6 (i.e. pTi368) and linked to 5′ *vir*B sequences, but not when located on pVCK225 (i.e. pSM368), which lacks these sequences.

Similar results are obtained for LR insertions located internal to *vir*B. The pSM1 and pSM26 carry inducible *vir*B insertions 1 and 26 within *Sal*I fragment 12 (Figure 2). The Tn*3*-HoHo1:: *Sal*I insertion fragment from each of these plasmids was subcloned to generate the respective deletion plasmids, pSM1cd and pSM26cd. In these plasmids the *Sal*I insertion fragment is no longer linked to the proximal region of *vir*B and the strains pTiA6(pSM1cd) and pTiA6(pSM26cd) do not display plant-inducible activity. Insertions 1 and 26 were also recombined from pSM1cd and pSM26cd onto pTiA6 to generate pTi1cd and pTi26cd. In these recombinant strains the proximal region of *vir*B is restored to insertions 1 and 26, and consequently their inducibility is restored as well (Table II). Thus >50% of the distal portion of *vir*B is fully dependent on proximal *vir*B sequences for its regulated expression.

Discussion

We have used 124 Tn*3*-HoHo1 insertions located across a 40-kb region of the A6 Ti plasmid to define the structural limits and respective mutant phenotypes of six *vir* complementation groups. Mutations in these loci eliminate (*vir*A, *vir*B, *vir*D and *vir*G) or restrict (*vir*C and *vir*E) the ability of *Agrobacterium* to infect plant cells. We note that while we have retained the nomenclature (i.e. *vir*A, *vir*B, etc.) previously used to designate the pTiA6 *vir* loci (Klee *et al.*, 1983), our assignments for the location and structural limits of each of the *vir* complementation groups are different from those reported earlier. Also *vir*G (and *pin*F) was not previously identified.

Since Tn*3*-HoHo1 contains the coding sequences of the *E. coli lac*Z gene, the regulated expression of the pTiA6 *vir* region sequences was characterized by measuring the levels of β-galactosidase expressed from each of the *vir*::Tn*3*-HoHo1 insertions under vegetative growth and cocultivation with plant cells. The results define the *vir* transcription units, their respective orientations and levels of expression as a function of growth condition, and show that each of the genetically defined *vir* loci corresponds to a defined *vir* transcription unit. *Vir*B, *vir*C, *vir*D and *vir*E are plant-inducible and significantly expressed only in plant-induced bacteria, whereas *vir*A is constitutively expressed in vegetative and plant-induced cells and non-inducible, and *vir*G is both constitutively expressed and plant-inducible. The pTiA6 *vir* region also carries another plant-inducible locus, *pin*F, which has no detectable role in virulence. Based on the genetic data presented here, the 5′ termini of the *vir*-encoded transcripts have recently been mapped in S1 nuclease protection studies (Das *et al.*, 1986; Stachel and Zambryski, 1986).

At least two *vir* loci, *vir*B and *vir*D, likely encode polycistronic messages. *Vir*B is ~9 kb in length and complements as a single locus, and its distal sequences are clearly dependent on proximal *vir*B sequences for their regulated expression. *Vir*D is 4.5 kb, and with the exception of the insertion mutant pTi304, *vir*D mutations complement as a single locus. We have isolated a wild-type insertion (insertion 324) in *vir*D; since this insertion bisects the *vir*D locus, the *vir*D message probably encodes two or more cistrons located to the left and the right of the map position of insertion 324.

The mutant phenotype, size and relative expressibility of a *vir* locus likely reflects the particular function that it specifies. For example, since mutations in different *vir* loci result in two distinct phenotypes, avirulence (*vir*A, *vir*B, *vir*D and *vir*G) or attenuated virulence (*vir*C and *vir*E), these two classes of loci respectively encode functions that are absolutely essential for, or increase the efficiency of, plant transformation by *Agrobacterium*. Essential functions might include those which regulate *vir* induction, or are responsible for the synthesis, transfer and integration of a T-DNA intermediate molecule; accessory functions might include bacterial production of phytohormones or plant cell wall degradation enzymes, whose actions on the plant cell could increase its susceptibility to *Agrobacterium* and thus the efficiency of T-DNA transfer. A large polycistronic locus (i.e. *vir*B or *vir*D) might specify a set of proteins that in concert mediate a complex step in the transformation process, such as the synthesis of the T-DNA intermediate molecule or the transfer of this molecule to the plant cell. Also, the highly expressed *vir* loci (i.e. *vir*B, *vir*E, *vir*G and *pin*F) might specify proteins required in high concentration, such as structural proteins, while the less expressed loci (*vir*A, *vir*C and *vir*D) might specify enzymatic functions.

A number of recent studies have begun to suggest some of the

functions encoded by particular *vir* loci. With regard to the accessory *vir* loci, *vir*C has been implicated in specifying, in part, the plant host range of different species of *Agrobacterium* (Yanofsky *et al.*, 1985). *Vir*E mutant strains, but not other *vir* strains, can be extracellularly complemented by coinfection with bacteria that carry a wild-type *vir* region (Otten *et al.*, 1984; D.Corbin and S.E.Stachel, unpublished results). Thus *vir*E might specify the production of a protein or compound that functions extracellularly or within the target plant cell during infection. With regard to the essential *vir* loci, *vir*A and *vir*G have recently been shown to be essential for the activation of *vir* expression in response to the plant signal molecule (Stachel and Zambryski, 1986), and these loci comprise the *vir* regulatory system. Therefore, of the essential *vir* loci, *vir*B and *vir*D are candidates for *vir* loci which specify the machinery that directly mediates the T-DNA transfer process. The present study provides an experimental foundation for the future identification of the specific functions encoded by these loci.

Materials and methods

Construction of plasmids

pVCK102, pVCK219, pVCK221 and pVCK225 (Knauf and Nester, 1982) carry overlapping segments of the pTiA6 *vir* region, and were previously constructed by cloning a *Sal*I partial digest of pTiA6 into the unique *Sal*I site of the broad-host range vector pVCK102. Here pVCK219, pVCK221 and pVCK225 were mutagenized in *E. coli* with Tn*3*-HoHo1 (Stachel *et al.*, 1985a) to generate a set of pSM(*vir*::Tn*3*-HoHo1) insertion plasmids. The position and orientation of the Tn*3*-HoHo1 insertions were determined by *Eco*RI, *Bam*HI and *Sal*I restriction analysis of plasmid DNA. In cases of ambiguity, *Hin*dIII, *Sst*I and *Kpn*I digests were also analyzed. The map position of each insertion was determined to an accuracy of at least ±0.20 kb. Because pVCK102 and Tn*3*-HoHo1 confer resistance to kanamycin and carbenicillin respectively, the insertion plasmids give resistance to both these antibiotics.

Tn*3*-HoHo1 is not cleaved by *Sal*I, and thus the *Sal*I::Tn*3*-HoHo1 fragment carried by a pSM(*vir*::Tn*3*-HoHo1) insertion plasmid can be independently isolated and cloned to produce a *Sal*I deletion derivative of the insertion plasmid. The insertion plasmid is digested with *Sal*I, ligated and *E. coli* transformants are selected on kanamycin and carbenicillin. Plasmid DNA from each of these transformants is subsequently screened with *Sal*I to identify pVCK102 clones that only carry the *Sal*I::Tn*3*-HoHo1 fragment. The deletion derivative of an insertion plasmid is designated pSM(X)cd, where X corresponds to the assigned number of the insertion of the parent plasmid. Plasmid conjugal transfer was by the method of Ditta *et al.* (1980), plasmid isolation was by the method of Birnboim and Doly (1979), and standard recombinant DNA procedures were as described by Maniatis *et al.* (1982).

Agrobacterium strains

Each pSM(*vir*::Tn*3*-HoHo1) insertion plasmid was conjugally transferred to *Agrobacterium* strain A348 (Garfinkel *et al.*, 1981), which carries pTiA6, to generate a corresponding A348(pSM*vir*::Tn*3*-HoHo1) merodiploid strain. Using the procedure of Garfinkel *et al.* (1981), the Tn*3*-HoHo1 insertion was homologously recombined onto pTiA6 to generate a corresponding pTiA6::Tn*3*-HoHo1 recombinant strain. Each recombinant strain also carries pPH1JI (Hirsch and Beringer, 1983), which confers gentamicin resistance and was used to select for the recombination event.

The avirulent *Agrobacterium* strains A1010, A1023 and A1061 are described by Garfinkel and Nester (1980). The position of the respective Tn*5* insertion mutation of A1010 and A1061 was determined from *Hpa*I restriction analysis (D. Garfinkel, personal communication), and of A1023 by *Bam*HI, *Eco*RI and *Hpa*I restriction analysis (S.E.Stachel, unpublished results). *Agrobacterium* strain AETW7 was constructed as follows. The *Eco*RI fragment 21 of pTiA6 (Figure 2) was subcloned onto pBR325. The resultant plasmid clone was opened at the *Bam*HI site of *Eco*RI fragment 21, treated with Klenow to blunt-end-fill this site and ligated to produce a plasmid carrying a 4-bp insertion mutation at the *Bam*HI site of *Eco*RI fragment 21 (S.E.Stachel, unpublished results). This (potential frameshift) mutation was subsequently recombined onto the Ti plasmid of the virulent Tn*5* strain A1085 (Garfinkel and Nester, 1980) to give the avirulent strain AETW7.

Bacterial and plant culture conditions

E. coli (pSM*vir*::Tn*3*-HoHo1) strains were grown at 37°C on L-agar and LB liquid media (Miller, 1972) containing 100 μg/ml each of carbenicillin and kanamycin. *Agrobacteria* were grown at 28°C on AB minimal agar, and in Mg/L and AB/glucose liquid media (Chilton *et al.*, 1974). For the pTiA6(pSM*vir*::Tn*3*-HoHo1) merodiploid strains, 100 μg/ml each of carbenicillin and kanamycin (Sigma) were included in the growth media, while for the pTiA6::Tn*3*-HoHo1 recombinant strains, 100 μg/ml of carbenicillin was used.

Cultures of a *N. tabacum* callus suspension cell line, designated NT1, were grown in 50 ml of Murashige and Skoog medium (Difco) supplemented with 0.2 mg/ml 2,4-dichlorophenoxyacetic acid (Sigma) in 250-ml flasks at 28°C and 120 r.p.m. as described by Stachel *et al.* (1986).

Determination of virulent phenotype

Each pTiA6::Tn*3*-HoHo1 recombinant strain was inoculated on *K. daigremontiana* leaf wounds, as described by Garfinkel and Nester (1980). All test leaves were also independently inoculated with the wild-type virulent parent A348(pPH1JI) and the Ti plasmidless A136(pPH1JI) control strains. Except for the presence of pTiA6 these two strains are isogenic, and except for the presence of Tn*3*-HoHo1 all the recombinant strains are isogenic with A348(pPH1JI). A348 induces undifferentiated tumors on *Kalanchoe* leaf wounds that are apparent within 10 days post-inoculation, and large tumors that cover the complete wound site are seen by 30 days. A136 is completely avirulent. Virulent phenotype was scored 15 and 60 days post-inoculation. Several strains were also tested on *N. glauca* stem wounds (Garfinkel and Nester, 1980) and with *N. tabacum* SR1 leaf discs (Horsch *et al.*, 1985).

Determination of vegetative and plant-induced β-galactosidase activity

Specific units of β-galactosidase activity were determined as previously described (Stachel *et al.*, 1985a) and are reported as U/bacterium. Vegetative activity was determined following overnight growth in AB/glucose liquid minimal medium. For plant-induced activity 2 ml of 2-day NT1 plant cell culture was inoculated with an overnight bacterial culture to an absorbance of 0.1 OD/ml at 600 nm/cm. Cocultivations were carried out at 28°C in 60-mm Petri plates. After 16 h, bacteria were separated from the plant cells using Miracloth® (Calbiochem) and their plant-induced activity determined. The vegetative and plant-induced activities of each strain were measured in duplicate.

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