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Regulation of the *vir* Genes of *Agrobacterium tumefaciens* Plasmid pTiC58

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The virulence (*vir*) region of pTiC58 was screened for promoter activities by using gene fusions to a promoterless *lux* operon in the broad-host-range vector pUCD615. Active *vir* fragments contained the strongly acetosyringone-inducible promoters of *virB*, *virC*, *virD*, and *virE* and the weakly inducible promoters of *virA* and *virG*. Identical induction patterns were obtained with freshly sliced carrot disks, suggesting that an inducer is released after plant tissue is wounded. Optimal conditions for *vir* gene induction were pH 5.7 for 50 μM acetosyringone or sinapic acid. The induction of *virB* and *virE* by acetosyringone was strictly dependent on intact *virA* and *virG* loci. An increase in the copy number of *virG* resulted in a proportional, acetosyringone-independent increase in *vir* gene expression, and a further increase occurred only if an inducing compound and *virA* were present.

Pathogenic *Agrobacterium tumefaciens* strains harbor large (approx. 200-kilobase [kb]) tumor-inducing (Ti) plasmids, which are responsible for crown gall tumor disease on susceptible host plants. Two regions of the Ti plasmids are required for pathogenicity: the T-DNA, which is transferred to and stably maintained in transformed plant cells, and the *vir* region, which may be involved in surface interactions between bacteria and plant cells and the processing, transfer, and integration of the T-DNA. A third region of Ti plasmids contains the genes for the catabolism of opines. Specific opines produced by transformed plant cells have been used to classify Ti plasmids. Two extensively studied groups of Ti plasmids are those encoding the synthesis and catabolism of octopine, such as pTiAch5, pTiA6, pTiB6S3, and pTi15955, and of nopaline, such as pTiC58 and pTiT37.

The *vir* region of octopine and nopaline Ti plasmids is composed of several complementation groups (13, 18, 22). In the octopine-type plasmids, these groups have been designated *virA*, *virB*, *virC*, *virD*, *virE*, *virF*, and *virG*. In the nopaline-type plasmid pTiC58, *virF* is not present (11). The counterparts of *virB* (T. Hirooka, R. C. Lundquist, P. M. Rogowsky, R. C. Tait, and C. I. Kado, submitted for publication), *virC* (formerly *bak* [3]), *virD* (formerly *hdv* [10]), *virE* (11, 12), and *virG* (B. S. Powell, G. K. Powell, R. O. Morris, P. M. Rogowsky, and C. I. Kado, Mol. Microbiol., in press) have been identified on the basis of sequence homologies. Nucleotide sequence comparisons of the *vir* region genes show considerable homologies between octopine and nopaline Ti plasmids, and deletions and insertions detected by hybridization analyses (7) seem to be confined primarily to the regions between these six complementation groups.

Two types of *vir* gene control have been demonstrated. Positive regulation via the VirA/G mechanism was shown to affect the expression of *virB*, *virC*, *virD*, *virE*, and *virG* of the octopine plasmid pTiA6 (33). These genes are normally expressed at intermediate (*virG*) or very low (*virB*, *virC*, *virD*, and *virE*) levels and show under laboratory conditions a 13- to 120-fold increase in gene expression (31, 32) after exposure to acetosyringone or plant exudates. The inducibility of *virG* by acetosyringone has recently been disputed and attributed to a change from AB glucose medium (pH 7.0) to K3 medium (pH 5.6) during the experimental induction (34). Both VirA and VirG are required for induction (33, 35), and on the basis of sequence homologies to other pairs of regulatory proteins (24, 26, 35), the *virA* product may function as a "sensor," detecting the presence of the inducer, while the *virG* product may function as a "regulatory protein," stimulating transcription of other *vir* genes. An independent, second control mechanism was shown to be abolished in the pleiotropic *ros* mutant (4). Compared with the wild type, the *ros* mutation leads to a 20- to 170-fold increase in the expression of *virC* and *virD* from pTiC58 or pTiA6, while not affecting the expression of other *vir* loci. The mutation is not located on the Ti plasmid and may represent a permanent "on" state of a repressor-type regulator (2, 4). A specific physiological role for the *ros* regulation during tumorigenesis has not yet been established.

This paper reports the in vivo response of *vir* genes of the nopaline plasmid pTiC58 during interaction with freshly wounded plant tissue assayed in a nondisruptive procedure. This was accomplished by measuring the production of light by using *vir* gene fusions to the bioluminescence genes (*lux*) of *Vibrio fischeri* (6). Analyses of pTiC58 *vir* gene inductions under various laboratory conditions are presented and compared with the results obtained with the octopine plasmid pTiA6. A copy number effect of *virG* on the expression of other *vir* genes is described, and its impact on a proposed model of *vir* gene induction is discussed.

MATERIALS AND METHODS

Strains and plasmids. The bacteria used in this study were *A. tumefaciens* NT1 (M.-D. Chilton) and LBA4301 Rec$^-$ Rif^r (R. A. Schilperoort) and *Escherichia coli* HB101 (H. W. Boyer) and DH1 (D. Hanahan). Plasmids are listed in Table 1 or described in the text. The transposon Tn*5* insertion sites in various *vir* genes of pTiC58 Tra^c harbored in plasmids pJK107, pJK104, pJK190, pJK125, pJK210, pJK195 (17),

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TABLE 1. Plasmids[a]

Plasmid	Genetic characteristic[b]	Reference
pTiC58	Nopaline Ti plasmid	21
pTiC58 Tra[c]	Transfer-constitutive derivative of pTiC58	17
pRK2013	Helper plasmid for conjugal transfer	D. Helinski
pJE347	*luxI*::Tn*5*	6
pCM47	Promoterless *cat*	This paper
pUCD4	*ori*pSa *ori*pBR322 Tc^r Ap^r Km^r (Gm^r)	5
pUCD5B	*ori*pSa *ori*pBR322 Tc^r Ap^r Km^r (Gm^r)	9
pUCD1002	*ori*pTiC58 *ori*pBR322 Ap^r Km^r (Gm^r) Km^r (Nm^r)	9
pUCD2001	*ori*pTAR *ori*pBR322 Ap^r Tc^r Km^r (Gm^r)	9
pUC206B	*cat* promoter probe, *ori*pSa *ori*pBR327 Ap^r Km^r (Nm^r)	4
pUCD1546	*cat* promoter probe, *ori*pSa *ori*pBR322 Ap^r Km^r (Gm^r)	This paper
pUCD615	*lux* promoter probe, *ori*pSa *ori*pBR322 Ap^r Km^r (Gm^r)	This paper
pUCD308	*ori*pSa *ori*pBR322 Ap^r Tc^r	This paper
pUCD309	*virG virC ori*pSa *ori*pBR322 Ap^r Tc^r	This paper
pSW170	pUC19Cm::pTiA6 *Kpn*I fragment 10 (*virA*)	S. Winans
pVK102::Sa113b	pVK102::pTiA6 *Sal*I fragment 13b (*virG*)	S. Winans
pCP13-143	pCP13::pTi15955 *Hin*dIII fragments 16–3 (*virABG*)	S. Farrand
pCP13-35	pCP13::pTi15955 *Hin*dIII fragments 21b–19b (*virABGC*)	S. Farrand
pCP13-57	pCP13::pTi15955 *Hin*dIII fragments 3–17 (*virGCD*)	S. Farrand
pCP13-13	pCP13::pTi15955 *Hin*dIII fragments 21c–22d (*virCDE*)	S. Farrand

[a] Nomenclature from E. M. Lederberg, Plasmid Reference Center, Stanford University, and Novick et al. (27).

[b] Km (Gm) and Km (Nm) designate Km^r genes that also encode resistance to gentamicin and neomycin, respectively; *ori* and a plasmid name indicate the source of the origin of replication.

pJK502 (22), pJK503, pJK504, pJK505, and pJK506 (11) are shown in Fig. 2, as are the insertion sites of a promoterless *cat* gene followed by a promoter-proficient neomycin phosphotransferase II (*npt* II) gene contained in plasmids pJK516 (11) and pJK523 (T. Hirooka). The marker exchange technique (11) was used to construct the *virG* mutant Ti plasmid pJK710 as follows. The 1.32-kb *Sal*I fragment of pEBS1 (D. Zaitlin, Ph.D. thesis, University of California, Davis, 1984), containing the *npt*II gene of Tn*5*, was cloned into the *Xho*I site within the *virG* gene contained in pUCD2-11K2 (5). The resulting plasmid, pUCD1558, which is essentially a subclone of pTiC58 with an insertion in *virG*, was mated into NT1(pTiC58 Tra^c) by using the helper plasmid pRK2013 (D. Helinski) for mobilization. Transconjugants were grown for more than 60 generations to allow recombination and subsequently mated with LBA4301 to separate the recombination products from pUCD1558. Transconjugants of the second mating that had lost the tetracycline resistance (Tc^r) marker of pUCD2-11K2 but retained the *npt*II marker (Nm^r) harbored a mutant pTiC58 Tra^c with an Nm^r determinant in *virG*. The same technique was used to generate five additional mutant Ti plasmids: pJK702 and pJK703 carry insertions of an Nm^r determinant with *Eco*RI ends (D. Zaitlin, thesis) in the two *Eco*RI sites located in *virC2* and *virD2*, respectively, while pJK718 and pJK719 have a promoterless *cat* gene followed by a promoter-proficient *npt* II gene or the Tc^r determinant of Tn*1721* inserted in the *Bgl*II site of *virA*, respectively. Plasmid pJK720 carries both the Tc^r determinant of pJK719 in *virA* and the *cat* gene plus the Nm^r determinant of pJK518 (11) in *virE*. Most *vir-lux* and *vir-cat* fusion plasmids shown in Fig. 2 are based on *Eco*RI, *Bam*HI, or *Bgl*II restriction fragments shown in the same figure. Exceptions are pUCD1108 (*Xba*I-*Eco*RI), pUCD626 (*Hpa*I-*Eco*RI), pUCD1168 (*Bam*HI-*Hpa*I), pUCD1176 (*Pvu*II-*Eco*RI), pUCD1169 (*Pvu*II-*Pvu*II), pUCD1170 (*Pvu*II-*Pvu*II), and pUCD1956 (*Kpn*I-*Bgl*II) and plasmids pUCD1506, pUCD1511, pUCD1512, pUCD1513 (12), pUCD206-1 (10), pUCD206-7, and pUCD206-8 (3), which have been described elsewhere. Plasmids pUCD308 and pUCD309 are *Bgl*II deletion derivatives of pUCD734-11K1 and pUCD734-11K2, respectively (5). They are essentially broad-host-range plasmids containing the right half (promoterless 5′ part of *virD*) and the left half (*virC* and *virG*) of *Kpn*I fragment 11, respectively.

Media and antibiotics. *A. tumefaciens* strains were grown in medium 523 and on 523 agar plates (15), while *E. coli* was grown in LB medium and on LB agar plates (25). Antibiotics were used at the following concentrations (micrograms per milliliter) for *A. tumefaciens* or *E. coli*: chloramphenicol, 3 or 20; gentamicin, 30 or 3; kanamycin, 30 or 20; neomycin, 30; rifampin, 50; and tetracycline, 4 or 20. The medium used for induction with acetosyringone (induction medium) was Murashige minimal organics medium (GIBCO Laboratories) supplemented with 12.5 mM potassium phosphate, pH 5.7 (or different pH, where indicated), and the appropriate antibiotics. The selective medium used for matings from *E. coli* to *A. tumefaciens* NT1 contained 2% sucrose and 7 g of K_2HPO_4, 2 g of KH_2PO_4, 1 g of $NaNO_3$, and 0.3 g of $MgSO_4 \cdot 7H_2O$ per liter, plus the appropriate antibiotic.

DNA manipulations. Plasmids from *A. tumefaciens* were isolated essentially as described by Kado and Liu (16). Plasmid isolation from *E. coli* and all other DNA manipulations were performed by the methods of Maniatis et al. (23). Enzymes were purchased from New England BioLabs or Boehringer Mannheim, except for T4 DNA ligase, which was prepared by R. C. Tait. Acetosyringone (3′,5′-dimethoxy-4′-hydroxyacetophenone) and sinapic acid (3′,5′-dimethoxy-4′-hydroxycinnamic acid) were obtained from Aldrich Chemical Co., and all other chemicals were from Sigma Chemical Co.

Bacterial transconjugation. For bacterial matings, freshly grown donor and recipient strains and, if necessary, a helper strain harboring pRK2013 were taken from agar plates, mixed together in a small area on a 523 agar plate with a toothpick, incubated overnight at 29°C, and streaked for single colonies on selective medium.

Induction with acetosyringone. Portions (3 ml) of induction medium (pH 5.7) were inoculated with 0.1 ml of an overnight culture in medium 523 and incubated for 24 h at 29°C. The cells grown were subsequently used to inoculate 30 ml of induction medium in a 300-ml Nephelo flask (sidearm flask) to an optical density of 10 Klett units (green filter, 10^8 cells per ml). The cultures were shaken at 25°C for approximately 12 h, until they reached an optical density of 40 Klett units (3×10^8 cells per ml). Half of each culture (i.e., 15 ml) was then transferred to a fresh Nephelo flask and supplemented with 30 μl of acetosyringone to a final concentration of 20 μg/ml. The remaining 15 ml received 30 μl of solvent (75% methanol) as a control. The cultures were further incubated at 25°C, and their light production and cell density were mea-

sured or culture samples (3 to 6 ml) were taken for chloramphenicol acetyltransferase (CAT) assays as described below. The following modifications were made for pH shift experiments. The preculture was grown in induction medium (pH 7.0). When the culture reached an optical density of 40 Klett units, it was divided into three equal portions that were harvested and suspended in 15 ml of induction medium at pH 7.0, 5.7, or 5.7 with acetosyringone.

Measurement of light. Light production of cultures growing in Nephelo flasks or on agar plates was measured in a luminometer. The luminometer was developed in collaboration with Beckman Instruments Inc. (Fullerton, Calif.) and is essentially a light-tight box containing a photomultiplier tube and a rack to hold Nephelo flasks or agar plates. Due to the configuration of a Nephelo flask and photomultiplier tube in the luminometer, a constant but unknown portion of the light from the flask was detected by the photomultiplier tube. This signal was digitally displayed in counts per minute representing the light detected. The linear relationship between light emitted by the bacteria and the representative units is lost above 2×10^6 cpm, and therefore neutral filters (Wratten filter; Eastman Kodak Co.) of 10, 1, and 0.1% transmittance were used to extend the accuracy range of the instrument.

CAT assay. The respective strains were grown as described, harvested 12 or 18 h after induction with acetosyringone, suspended in 0.5 ml of assay buffer (50 mM Tris chloride, pH 7.8, 50 μM dithiothreitol), and lysed with a MICROSON ultrasonic cell disruptor (Heat Systems-Ultrasonics Inc, Formingdale, N.Y.). The photometric enzyme assay was performed as described earlier (29).

Carrot assay. Fresh carrot roots were peeled, surface sterilized by immersion for 30 s in 95% ethanol and 5 min in 5% sodium hypochlorite, cut into equal-sized disks with a sterile knife, and scooped on their basal side with a sterile melon scooper. Bacteria were grown as described for induction with acetosyringone. However, instead of inducing the bacteria with acetosyringone, they were harvested, suspended in 0.1 ml of induction medium without acetosyringone, and spread over the basal surface of the carrot disks. The disks were then placed (basal side up) on filter paper soaked with sterile water and photographed without an external light source with hypersensitive 35-mm astronomy film (Lumicon Inc., Livermore, Calif.). Exposure times were 12 to 14 h with a 35-mm lens and an aperture of f1.4.

Virulence assay. Inoculum preparation and virulence assays on stems of *Datura stramonium* (Jimson weed) have been described previously (19).

RESULTS

Construction of the promoter-probe plasmid pUCD615. To study the expression of *vir* genes in vivo during infection of wounded plant tissue, we constructed the plasmid vector pUCD615 (Fig. 1). This plasmid was designed to fuse a promoter of interest to bacterial bioluminescence genes (*lux*) so that promoter activity could be measured by the amount of light produced by the bacterial cell without disrupting the interaction between bacteria and plant tissue. The source of the *lux* genes was a subclone of plasmid pJE347 (6) that was kindly provided by J. Engebrecht. Plasmid pJE347 carries a Tn*5* insertion in the regulatory *luxI* gene, separating the five structural genes *luxC*, *luxD*, *luxA*, *luxB*, and *luxE* from their promoter, which is located upstream of *luxI*. In several cloning steps, the 7.6-kb fragment from the *Hpa*I site in the inverted repeat of Tn*5* to the *Sal*I site downstream of *luxE* was combined with the 9.9-kb *Eco*RI-*Bam*HI fragment of plasmid pUCD5B (9) and part of the pUC19 polylinker. The resulting plasmid, pUCD615, contains a promoterless *lux* operon with a multiple cloning site located about 600 base pairs (bp) upstream of the start codon of *luxC*. Unique restriction sites for *Bam*HI, *Eco*RI, *Sma*I, and *Xba*I as well as two closely linked *Sal*I sites allow the cloning of a large variety of promoter-active fragments. Functional fusions are limited to transcriptional fusions, because there is at least one translational stop codon in each open reading frame of the Tn*5* leader sequence (Fig. 1). Additional stop codons may be present in the unknown nucleotide sequence of the 3′ remnant of *luxI*. In addition to two selectable markers, which confer resistance to ampicillin and kanamycin-gentamicin, and a phage lambda *cos* sequence, the plasmid contains two origins of replication (*ori*). The broad-host-range *ori* of pSa allows replication in *A. tumefaciens* and many other gram-negative bacteria, and the *ori* of pBR322 gives rise to a high copy number in *E. coli*. The basis of mobilization (*bom*) site near the pBR322 origin (Fig. 1) allows the mobilization of pUCD615 by a number of R factors when *tra* functions are provided in *trans*. Thus, pUCD615 can be used to study promoter activities in a variety of bacterial species.

Genetic organization of the *vir* region of pTiC58. To study the expression of the pTiC58 counterparts of octopine Ti plasmid *vir* genes, the respective loci were first located relative to the physical map of pTiC58 by using nucleotide

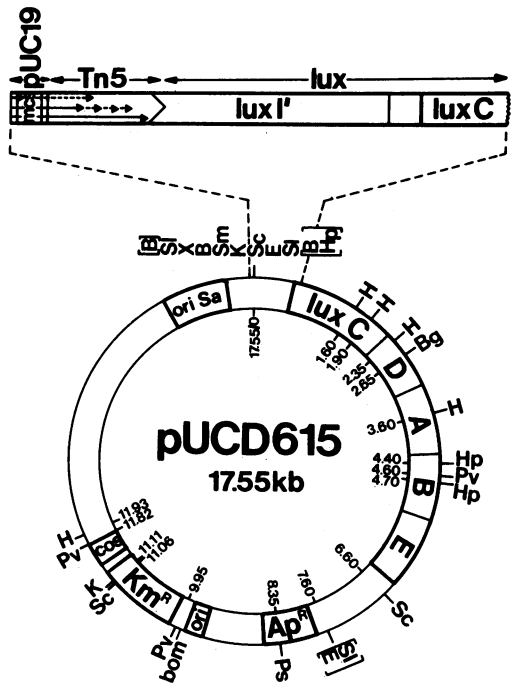

FIG. 1. Restriction map of pUCD615. The region between the multiple cloning site (mcs) originating from pUC19 and the *lux* genes is enlarged. Also indicated are translational stop codons in the remaining part of Tn*5* (by arrows), the remnant 3′ end of a regulatory *lux* gene (by *luxI'*), and the 5′ portion of the five structural *lux* genes (by *luxC*). Restriction endonuclease sites lost due to heterologous cloning steps are shown in brackets. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; K, *Kpn*I; Ps, *Pst*I; Pv, *Pvu*II; Sc, *Sac*I; Sl, *Sal*I; Sm, *Sma*I; X, *Xba*I; *cos*, packaging site of phage lambda; *ori*Sa, origin of replication from plasmid pSa; *ori*, origin of replication from pBR322; *bom*, basis of mobilization. Distances are shown in kilobase pairs (kb).

TABLE 2. Tumorigenic complementation of pTiC58 *vir* region mutations by overlapping clones of the pTi15955 *vir* region

pTiC58 *vir* mutant	Complementation[a] by plasmid:				
	pCP13-143 (*virA*$^+$*B*$^+$*G*$^+$)	pCP13-35 (*virA*$^+$*B*$^+$*G*$^+$*C*$^+$)	pCP13-57 (*virG*$^+$*C*$^+$*D*$^+$)	pCP13-13 (*virC*$^+$*D*$^+$*E*$^+$)	None
pJK270	+ + +	+ + +	+ + +	+ + +	+ + +
pJK107	+ + +	+ + +	−	−	−
pJK502	+ +	+ +	−	−	−
pJK190	+ + +	+ + +	−	−	−
pJK104	+ + +	+ + +	−	−	−
pJK125	+ + +	+ + +	−	−	−
pJK210	+ + +	+ + +	−	−	−
pJK710	+ + +	+ + +	+ + +	−	−
pJK702	+	+ + +	+ + +	+ +	+
pJK503	+	+ + +	+ + +	+ +	+
pJK195	−	−	+ + +	+ + +	−
pJK703	−	−	+ + +	+ + +	−
pJK504	−	−	+ + +	+ + +	−
pJK516	+ + +	+ + +	+ + +	+ + +	+ + +
pJK505	(+)	(+)	(+)	+ + +	(+)
pJK506	(+)	(+)	(+)	+ + +	(+)

[a] Symbols: + + +, full-size wild-type tumors; + +, large tumors, but detectably smaller than wild-type; +, small tumors covering the inoculation site; (+), occasional tumorous growth not covering the inoculation site; −, no sign of tumors. All strains were inoculated at two sites on two plants.

sequence comparisons with pTiA6, hybridization experiments with pTiAch5 (7), and complementation analysis with subclones of pTi15955 (Table 2). The locations of *virC*, *virG*, and *virE* (Fig. 2) were determined by homologies in the predicted amino acid sequences of 79% (3), 80% (Powell et al., in press), and 70% (12) to the respective loci of pTiA6. The complementation of the mutant Ti plasmids pJK503, pJK702 (both *virC*), pJK710 (*virG*), pJK505, and pJK506 (both *virE*) with the proper subclones of an octopine plasmid (Table 2) confirmed the locations shown in Fig. 2. In the case of *virD*, only the nucleotide sequences of the 5′ ends of the operons were published for both plasmids (10, 36) and show a DNA homology of 79% in the first 1,000 bp. Complementation analysis with mutant subclones of pTiC58 (T. Hirooka, Ph.D. thesis, University of California, Davis, 1986) shows that *virD* is an operon and extends at least to insertion JK504 (inner boundary of *virD* in Fig. 2), while the complementation of pJK195, pJK703, and pJK504 with only pCP13-57 and pCP13-13 (Table 2) confirms the homology to *virD* of the octopine plasmids. In the case of *virA*, the nucleotide sequence of 200 bp at the right end of the *Bgl*II fragment B (Fig. 2) was determined (one strand only, data not shown). It showed an 84% homology to bp 1364 to 1564 of the *virA* open reading frame (ORF) of pTiA6 and an 82% homology in the predicted amino acid sequence (20). Our location of this locus in pTiC58 (Fig. 2) was based on the assumption that *virA* has a very similar size in pTiA6 and pTiC58, as was the case for the other ORFs analyzed. This assumption was substantiated by the assignment of mutations JK107 and JK502 to different complementation groups (22), by the hybridization of the entire stretch of DNA to *virA* of an octopine plasmid (7) (Fig. 2), and by the location of *tzs* (1), which defines the maximal size of the proximal end of *virA*. The location of *virB* is based on complementation of the mutations JK502, JK190, JK104, JK125, and JK210 (inner boundaries of *virB* in Fig. 2) by the cosmid clones pCP13-143 and pCP13-35 but not by pCP13-57 or pCP13-13 (Table 2). Therefore, the mutations mapped in a region of pTiC58 which is homologous to either *virB* or *virA* or a region to the left of *virA* (Fig. 2). The locations of the mutations between *virA* and *virG* of pTiC58 and the hybridization of *Bgl*II fragment C of pTiC58 to *Hin*dIII fragment 3 and to *Eco*RI fragments 29a and 30b of pCP13-35 (T. Hirooka et al., submitted) showed that these mutations were part of the *virB* locus of this plasmid. The maximal size of the locus (outer boundaries in Fig. 2) was derived from the extent of hybridization between this region and *virB* of pTiAch5 (7) (Fig. 2).

Transcriptional activity of the *vir* region. Fusions to the *lux* operon were used to examine selected fragments of the *vir* region for promoter activity. Nineteen fragments of the *vir* region (Fig. 2) were cloned into pUCD615 (Fig. 1). Due to two possible orientations of some fragments, a total of 31 clones were analyzed. All of these fusion plasmids, in addition to pUCD607, which carries a *tet-lux* fusion (28), and the vector pUCD615 by itself were mobilized into *A. tumefaciens* LBA4301(pTiC58). Transconjugants were analyzed for light production in the presence and absence of acetosyringone. The bacteria were grown in induction medium, and light production and cell density were monitored every 3 h. The light production fell into three different categories: constitutive light production, which included the three strains harboring pUCD1193, pUCD1195, or pUCD1502; acetosyringone-induced light production, which included the 12 strains harboring pUCD626, pUCD1105, pUCD1106, pUCD1168, pUCD1172, pUCD1173, pUCD1186, pUCD1187, pUCD1194, pUCD1509, pUCD1510, or pUCD1512; and background levels of light production, which included the remaining 16 strains.

From the genetic map of the pTiC58 *vir* region (Fig. 2) and the available nucleotide sequence data (3, 10, 12; Powell et al., in press; and the partial *virA* sequence mentioned above), we concluded that 11 of the 15 light-producing (positive) strains contained fusions to one of the six *vir* promoters as follows (the numbers refer to the first base pair of the respective ORF): pUCD1186 and pUCD1509 at bp 1564 of *virA*, pUCD1195 at bp 14 of *virG*, pUCD1168 at bp 344 of *virC1*, pUCD1172 at bp 7 of *virC2*, pUCD626 and pUCD1173 at bp 678 of *virD2*, pUCD1194 at bp 5 of ORF3 of *virE*, and pUCD1512 between ORF1 and ORF2 of *virE*. The fusion sites of pUCD1187 and pUCD1105 both mapped in a region that hybridized in Southern blots to the *virB* locus of the octopine plasmid pTi15955 (T. Hirooka et al., submitted).

The remaining four positive plasmids carried the following

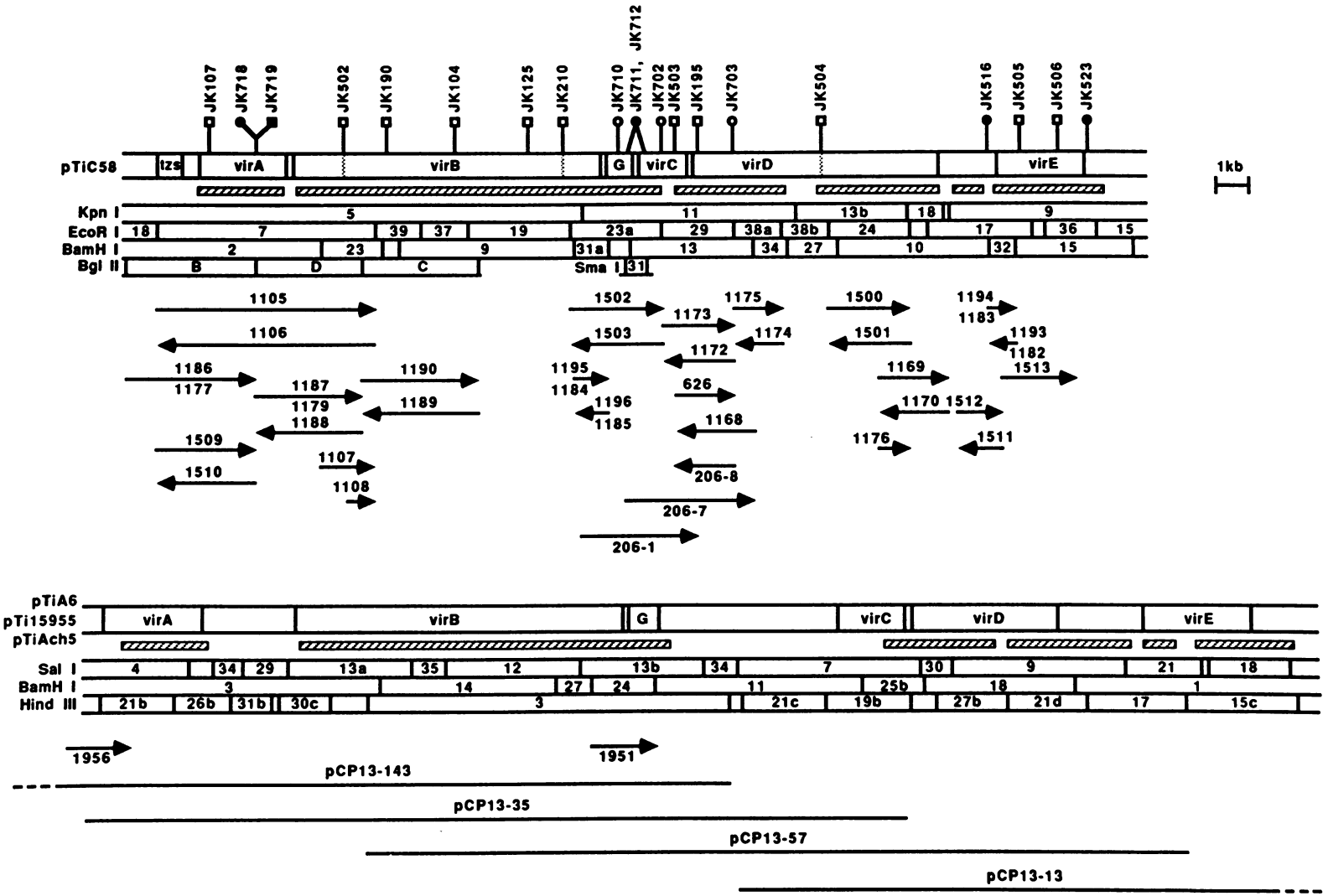

FIG. 2. Genetic organization of nopaline and octopine Ti plasmid *vir* regions. The locations of the six *vir* loci and *tzs* (pTiC58 only) are shown above the restriction map of pTiC58 (top) and the equivalent maps (bottom) of pTiA6 (31), pTi15955 (24), and pTiAch5 (13). Minimum (dashed lines) and maximum (solid lines) sizes of *virB* and *virD* of pTiC58 are based on assumptions described in the text. Hatched boxes indicate regions of homology between pTiC58 and pTiAch5 and are adopted from Engler et al. (7). Insertion sites of Tn*5* (□), Nm^r determinants (○), Tc^r determinants (■), and promoterless *cat* genes followed by promoter-proficient Nm^r determinants (●) are also shown. In the case of JK711 and JK712, the 0.41-kb *Sma*I fragment 31 of pTiC58 was replaced by a promoterless *cat* gene followed by a promoter-proficient Nm^r determinant. The arrows below the maps represent restriction fragments (and orientations) that were cloned into the promoter-probe plasmids pUCD615 (*lux* fusions, numbers above the arrows) and pUCD206B (*cat* fusions, numbers below the arrows), with the arrowheads representing the transcriptional fusion sites: $\xrightarrow{vir}$ [*lux*]. The numbers are the pUCD numbers of the resulting fusion plasmids. The restriction endonuclease sites used during the construction are listed in Materials and Methods, and the exact fusion sites of promoter-active fragments are mentioned in the text. Cosmid clones of pTi15955 are shown at the bottom.

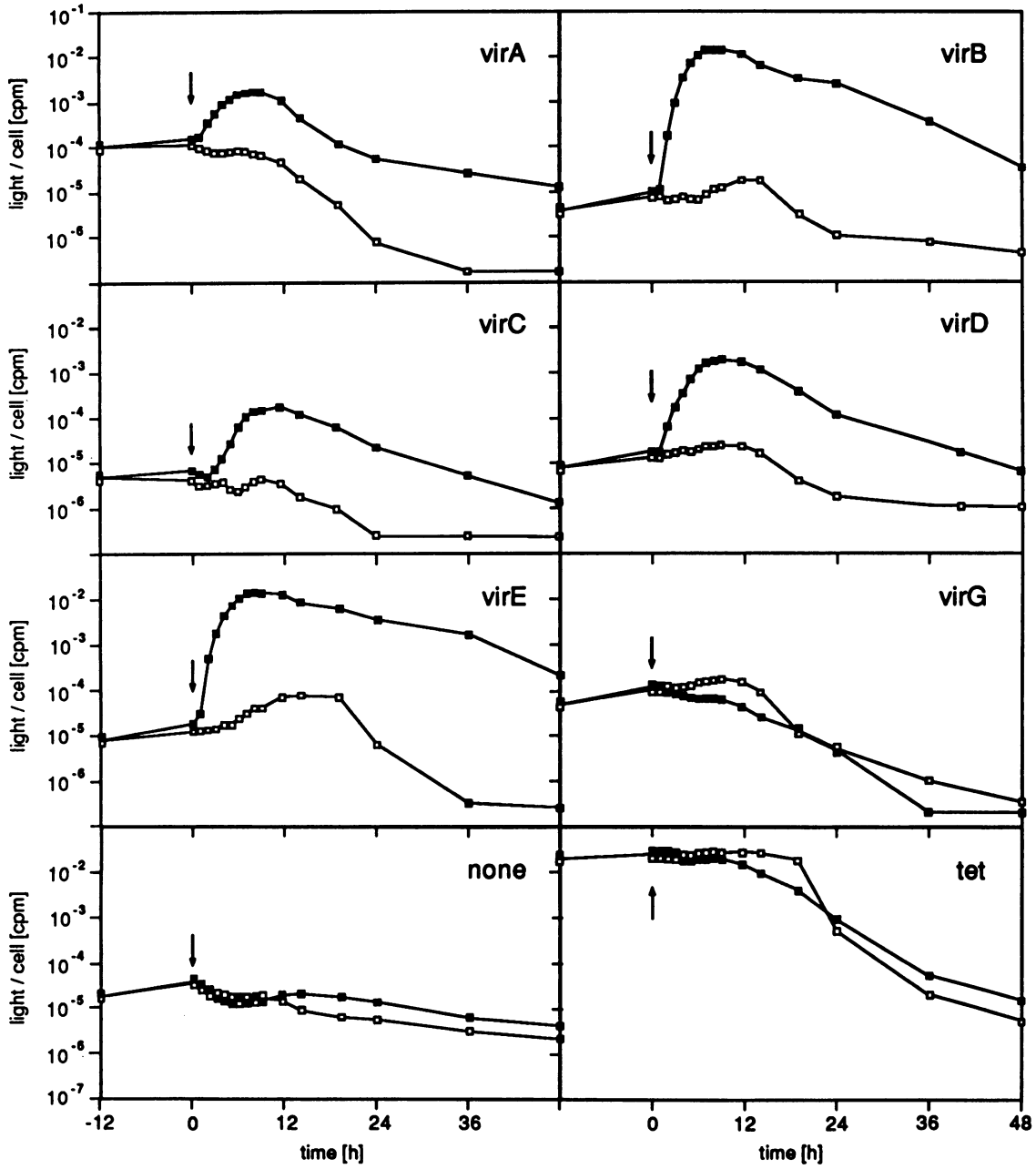

FIG. 3. Induction of *vir-lux* gene fusions with acetosyringone. The light production of LBA4301(pTiC58) harboring different *vir-lux* fusion plasmids is shown. The vertical axis is a logarithmic scale of the detected counts per minute per cell. The bacteria were grown with (■) and without (□) 100 μM acetosyringone added at time zero (vertical arrow). The strains used contained the following indicator plasmids: pUCD1186 (*virA-lux*), pUCD1187 (*virB-lux*), pUCD1168 (*virC-lux*), pUCD1173 (*virD-lux*), pUCD1194 (*virE-lux*), pUCD1195 (*virG-lux*), pUCD615 (none-*lux*), or pUCD607 (*tet-lux*). Each fusion was assayed at least twice.

promoters. pUCD1193 carried a fusion to a promoter linked to *virE* but reading in the opposite direction from *virE* transcription (right to left in Fig. 2) (12) and showed a slight decrease in expression after addition of acetosyringone (15×10^{-6} versus 56×10^{-6} cpm/cell). Similarily, pUCD1502 had a fusion in the ORF of *virC2*, but to a promoter reading in the opposite direction, and also showed a slight decrease (34×10^{-6} versus 86×10^{-6} cpm/cell). The fusion sites of pUCD1106 and pUCD1510 were at bp 724 of *tzs* (1), a gene located to the left of the *vir* region, which is probably not required for tumorigenesis and encodes *trans*-zeatin synthase, an enzyme needed for the synthesis of cytokinin. The fusion showed a strong increase in expression in the presence of acetosyringone (2.4×10^{-2} versus 1.1×10^{-4} cpm/cell for both plasmids).

The negative group encompassed fusions of internal *virB* (pUCD1107, pUCD1108, and pUCD1190) and internal *virD* fragments (pUCD1175). All these results are consistent with the genetic organization of the pTiC58 *vir* region presented in Fig. 2.

Because different fusions to a given *vir* promoter behaved similarly to each other, only one clone from each *vir* pro-

TABLE 3. Comparison of *vir-cat* and *vir-lux* fusions[a]

Gene fused	*vir-cat* plasmid	CAT activity (nmol/min per mg of protein)		*vir-lux* plasmid	Luciferase activity (μcpm/cell)		Fold induction	
		With acetosyringone	Without acetosyringone		With acetosyringone	Without acetosyringone	CAT	Luciferase
virA	pUCD1177	223	67	pUCD1186	1,600	69	3.3	23
virB	pUCD1179	1,002	4.8	pUCD1187	14,800	13	209	1,140
virC	pUCD206-8	127	<0.4	pUCD1168	138	<5	>318	>28
virD	pUCD206-7	550	11	pUCD1173	1,630	23	51	71
virD[b]	pUCD206-1	1,544	51				30	
virE	pUCD1183	990	30	pUCD1194	13,200	41	33	322
virG	pUCD1184	113	133	pUCD1195	56	156	0.85	0.36
None	pUCD206B	3.8	3.4	pUCD615	16	17	1.12	0.94

[a] Enzyme activities were measured 18 h after induction. The lowest levels of detection were 0.4 nmol/min per mg for CAT activity and 5 μcpm per cell for luciferase activity.
[b] Plasmid also contained *virG*.

moter was analyzed in further detail. To monitor induction kinetics, the number of light measurements was increased during early induction. Without acetosyringone, the *lux* genes fused to *virA* or *virG* were expressed at a substantial basal level. Basal expression was barely detectable in fusions to the *virB*, *virC*, and *virD* promoters, whereas the basal expression of the *virE* fusions ranged between those of the other two groups. In most cases the presence of acetosyringone led to dramatic increases in light production in times as short as 3 to 6 h (Fig. 3). During this time, strains with *virB* and *virE* fusions reached very high light levels compared with the control cultures without inducer, while the light levels were intermediate in *virA* and *virD* fusions and low in a *virC* fusion (Fig. 3). However, the *virC* fusion ranged between the *virA* and *virD* fusions in light production when the ratios of light produced in the presence and absence of acetosyringone (fold induction in Table 3) were compared. No significant change in light production was observed after addition of acetosyringone for the *virG* fusion and in the control strains harboring pUCD607 and pUCD615. The decrease in light production after approximately 18 h was common to all strains and was probably due to changing physiological conditions that occurred as the bacteria entered the stationary phase.

Because the *lux* genes of *V. fischeri* had not been used before for quantitative comparisons of different promoter activities, we wanted to compare the luminescence data (Fig. 3) with CAT activities by using the same *vir* promoter fragments fused to *cat*. The fusion plasmids pUCD1177 (*virA*), pUCD1179 (*virB*), pUCD206-8 (*virC*), pUCD206-1 and pUCD206-7 (both *virD*), pUCD1183 (*virE*), and pUCD1184 (*virG*) (Fig. 2) were used to transform LBA4301(pTiC58). Transformants were grown in the presence or absence of acetosyringone under standard induction conditions, and their CAT activities were determined 18 h after induction. The results (Table 3) qualitatively resembled the induction data obtained with *lux* fusions; *virB*, *virC*, *virD*, and *virE* were highly induced, while *virA* responded only weakly and *virG* not at all to acetosyringone.

To show that the increase in light production was strictly dependent on the presence of pTiC58 in addition to the indicator plasmid, the respective *lux* fusion plasmids were mobilized into *A. tumefaciens* LBA4301 harboring no pTiC58 plasmid, and the transconjugants were analyzed for light production in the presence and absence of acetosyringone. None of the fused promoters responded to acetosyringone in the absence of pTiC58, and light production was essentially the same as for LBA4301(pTiC58) harboring the same *lux* fusion plasmids in the absence of inducer (data not shown).

Regulation of *virA* and *virG* in pTiC58. The induction properties of *virA* and *virG* in pTiC58 differed from those

TABLE 4. Expression of *virA* and *virG*

Fusion plasmid	Gene fused	Helper plasmid	*vir* genes in *trans*	CAT activity[a] (nmol/min per mg of protein)			Fold induction at pH 5.7
				With acetosyringone, pH 5.7	Without acetosyringone, pH 5.7	Without acetosyringone, pH 7.0	
pJK718	*virA*	pUCD1105	*virA*	134	52	n.d.[b]	2.5
		pUCD1187	None	50	51	n.d.	1.0
pJK720	*virE*	pUCD1105	*virA*	1,065	38	n.d.	28.3
		pUCD1187	None	29	28	n.d.	1.0
pJK712	*virG*	pUCD309	*virG*	168	95	78	1.8
		pUCD308	None	100	88	n.d.	1.1
pJK711	*virC*	pUCD309	*virG*	132	4	n.d.	30.0
		pUCD308	None	1	1	n.d.	0.9
pUCD1177	*virA*	pTiC58	All	186	77	n.d.	2.4
pUCD1956	*virA*[c]	pTiC58	All	48	34	n.d.	1.4
pUCD1184	*virG*	pTiC58	All	113	89	63	1.3
pUCD1951	*virG*[c]	pTiC58	All	234	87	64	2.7

[a] Measured 12 h after induction. Numbers represent means of duplicate determinations.
[b] n.d., Not determined.
[c] *vir* genes of pTiA6.

TABLE 5. Effect of environmental parameters on the induction of *virE*[a]

Expt type and change or addition to medium[b]	Light production (μcpm/cell)		
	With acetosyringone	With sinapic acid	Without inducer
pH (with 50 mM K-PO_4)			
5.5	6,270	3,694	14
5.7	11,809	7,779	20
6.0	10,772	5,562	14
7.0	888	36	22
Inducer (μM)			
1	488	1,018	31
5	2,177	2,657	31
10	4,297	3,807	31
50	7,738	5,550	31
100	4,262	5,199	31
250	3,039	4,017	31
Supplement			
None	3,891	n.d.[c]	26
Casein hydrolysate (0.8%)	8,103	n.d.	39
Yeast extract (0.8%)	12,115	n.d.	17
Trp, Tyr, Phe (100 μM each)	4,327	n.d.	18
Plant medium			
With K-PO_4	3,996	n.d.	28
With MES	3,577	n.d.	29
Minimal medium			
With K-PO_4	7,559	n.d.	7
With MES	3,525	n.d.	46

[a] Determinations were made 6 h (pH experiments) or 12 h (all other experiments) after induction.

[b] K-PO_4, Potassium phosphate. Plant medium was Murashige and Skoog medium (see Materials and Methods) with 12.5 mM MES or potassium phosphate, pH 5.7. Minimal medium contained 3% sucrose, NH_4Cl (1 g/liter), and $MgSO_4 \cdot 7H_2O$ (0.3 g/liter) with 12.5 mM MES or potassium phosphate, pH 5.7.

[c] n.d., Not determined.

obtained for the octopine plasmid pTiA6 (31, 32). While the *virA* gene of pTiA6 was not inducible, its pTiC58 counterpart was induced at least 3.3-fold (*cat* fusion) and possibly 23-fold (*lux* fusion) by acetosyringone (Table 3). In contrast, *virG* was induced 13-fold in pTiA6 but not in pTiC58. Because the upstream regions of the pTiA6 cosmids used in the previous study (31) were larger than those of the pTiC58 subclones (11.8 versus 2.2 kb for *virA* and 2.0 versus 0.8 kb for *virG*), it is possible that the subcloned fragments lacked some regulatory sequences or that induction in the intact Ti plasmid occurred by readthrough from other promoters. In particular, transcription of *virG* from the strongly inducible *virB* promoter located 9 kb upstream was a distinct possibility. To test the expression of *virA* and *virG* in their native genetic environment on an intact Ti plasmid, the *cat* fusions JK718 (*virA-cat*) and JK712 (*virG-cat*, Fig. 2) were constructed and transferred to LBA4301. Because the resulting mutant Ti plasmids lacked an intact *virA* or *virG* locus, these genes were provided in *trans* on plasmid pUCD1105 (*virA*) or pUCD309 (*virG*), respectively. In the case of *virA*, a 2.6-fold induction by acetosyringone was observed (Table 4), which was very similar to the 3.3-fold induction observed with the isolated fragment. In contrast, the marginal 1.8-fold induction of *virG* was different from both the slight decrease in expression found with the subcloned *virG* promoter fragment (Table 3) and the 13-fold induction observed with the pTiA6 counterpart. The complementation of *virA* and *virG* worked well, because strains carrying pUCD1105 and pJK720 (*virA-tet*, *virE-cat*) or pUCD309 and pJK711 (Δ*virG*, *virC-cat*) were induced to expected levels (Table 4). Plasmids pUCD1187 and pUCD308, which had the same vector portion as pUCD1105 and pUCD309 but lacked intact *virA* or *virG* loci, did not complement the mutations (Table 4).

The pTiA6 *virA* and *virG* genes, including 0.9 and 1.2 kb of upstream sequences, respectively, were subcloned in pUCD206B. The resulting plasmids, pUCD1956 (*virA-cat*) and pUCD1951 (*virG-cat*) (Fig. 2), were transferred to LBA4301(pTiC58), and gene expression was monitored under standard induction conditions. In the case of *virA* the basal level of the pTiA6 gene was about half that of the pTiC58 gene, and its marginal (1.4-fold) induction was definitely lower than the 2.4-fold induction of its counterpart from pTiC58 (Table 4). In contrast, the basal levels of the two *virG* genes were approximately the same, and the 2.7-fold induction of the pTiA6 gene was higher than the 1.3-fold induction of the gene from pTiC58. However, the 13-fold induction of the pTiA6 gene seen in Tn*3*Hoho1 insertions could not be reproduced.

Since the 13-fold induction of *virG* observed by Stachel et al. (31, 32) was recently attributed to a medium pH change from 7.0 to 5.7 (34), the expression of both *virG* fusions was measured after a pH shift (see Materials and Methods). A slight increase in expression was observed when *A. tumefaciens* cells were shifted from pH 7.0 to 5.7 (Table 4). However, the total induction (pH 5.7 with acetosyringone versus pH 7.0 without acetosyringone) was only 1.8-fold for *virG* of pTiC58 and 3.6-fold for *virG* of pTiA6.

Induction conditions. The *vir* gene induction conditions used throughout this study (see Materials and Methods) were essentially those of Stachel et al. (30). Induction studies were extended to include parameters such as the effect of a second inducing compound and different types of induction medium (Table 5). The pH optimum for induction with either acetosyringone or sinapic acid was pH 5.7. Experiments with different concentrations of either inducer

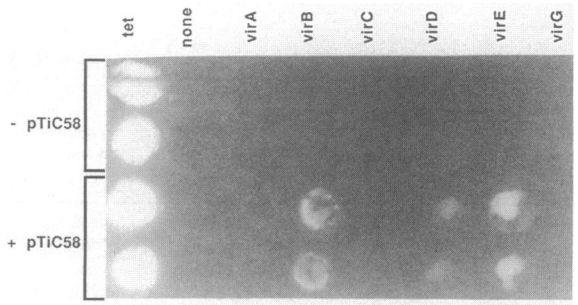

FIG. 4. Expression of *vir* genes on carrot disks. Strains LBA4301 (−pTiC58) and LBA4301(pTiC58) (+pTiC58) harboring the promoter-*lux* fusion plasmids pUCD607 (*tet*), pUCD615 (none), pUCD1186 (*virA*), pUCD1187 (*virB*), pUCD1168 (*virC*), pUCD1173 (*virD*), pUCD1194 (*virE*), and pUCD1195 (*virG*) were spotted on carrot disks in duplicates. The carrots were photographed without external light as described in Materials and Methods. The 24-h exposure for the detection of bacterial light was started 16 h after inoculation of the carrot disks. Sectored light emission is due to uneven scooping of the carrot surface. The experiment was repeated with duplicates of each fusion.

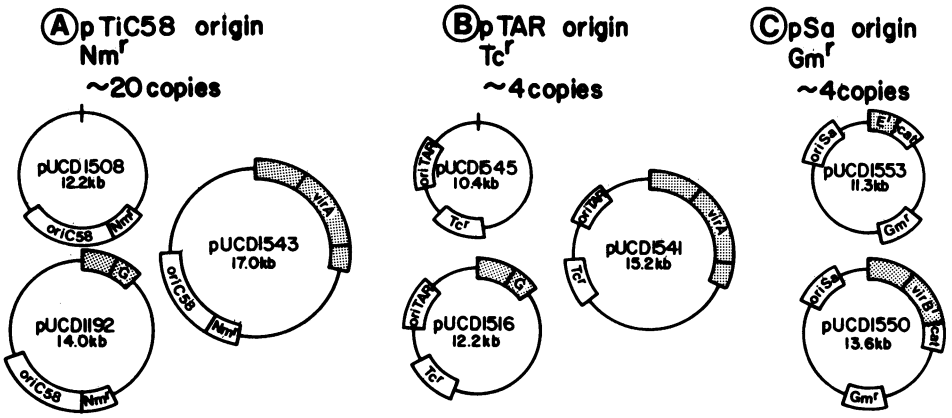

FIG. 5. Plasmids used to study the role of *virA* and *virG* in induction. High-copy-number plasmid (A) and low-copy-number plasmid (B) subclones of *virA* and *virG* were constructed as follows. The *virA*-containing *Eco*RI fragment 7 (Fig. 2) was digested with *Bam*HI, and the ends of the resulting large subfragment (4.8 kb; shaded) were converted to *Kpn*I sites. The starting material for *virG* was *Kpn*I fragment 11 (Fig. 2); a *Kpn*I linker was inserted in the *Pvu*II site at the end of *virC2*. The resulting 1.8-kb *Kpn*I fragment carrying *virG* (shaded), the 4.8-kb *Kpn*I fragment carrying *virA* (shaded), and an *Sal*I linker were separately cloned in the unique *Kpn*I site of pUCD1002 (9), leading to the three plasmids pUCD1192 (*virG*), pUCD1543 (*virA*), and pUCD1508 (control). (A) This set of plasmids conferred neomycin resistance to the bacterial host and were based on a mutant pTiC58 origin of replication with a deletion in the copy number locus (8); therefore, these plasmids were present at approximately 20 copies per cell in *A. tumefaciens*. (B) The same *Kpn*I fragments with *virG*, *virA*, and a *Bam*HI linker were used to construct the plasmids pUCD1516 (*virG*), pUCD1541 (*virA*) and pUCD1545 (control). The vector in these cases was pUCD2001 (9). This plasmid harbors a Tc^r gene and is based on the pTAR origin of replication, which is compatible with pTiC58 and has a copy number of approximately four in *A. tumefaciens*. To measure the effects of these *virA* and *virG* clones, the indicator plasmids pUCD1550 and pUCD1553 (C) were constructed. They carry a Gm^r gene and the pSa origin of replication (four copies per cell), which is compatible with both pTiC58 and pTAR. They are derivatives of a promoter-probe plasmid, pUCD1546, which was constructed by replacing the 3.3-kb *Bam*HI-*Pst*I fragment of pUCD4 (5) with the 1.53-kb *Bam*HI-*Pst*I fragment of pCM47. Plasmid pCM47 was made by exchanging the *Eco*RI fragments of pCM4 and pCM7 (T. Close) and contains a promoterless *cat* gene and the part of the *bla* gene needed to restore Ap^r in the resulting plasmid, pUCD1546. The *Bam*HI site 38 bp upstream of *cat* was used to clone the 3.1-kb *Bgl*II fragment D (shaded, see Fig. 2) and the 0.8-kb *Bam*HI fragment 32 (shaded, see Fig. 2) of pTiC58 so that the *virB* (pUCD1550) and *virE* promoters (pUCD1553) were fused to *cat*. In addition to the markers shown, all plasmids carried the pBR322 origin of replication (including the *bom* site) and an Ap^r marker.

showed maximum induction activity at 50 μM. The addition of potential competitors of the inducing compounds, such as aromatic amino acids, yeast extract, and casein hydrolysate, did not interfere with induction. Induction was also unaffected by a change in the buffer agent (potassium phosphate versus MES [morpholineethanesulfonic acid]) or when the Murashige and Skoog plant medium was replaced by a simple bacterial medium. Similar results were obtained in single experiments with *virB-lux* and *virC-lux* fusions (data not shown).

Expression of *vir* genes on carrot disks. *lux* fusions, unlike fusions to *lacZ* or *cat*, allow one to measure gene expression without disrupting the bacterial cells or interfering with interactions between the bacterial pathogen and its plant host. We examined *vir* gene induction during tumorigenesis on wounded plant tissue. Strains LBA4301 and LBA4301 (pTiC58) with the same eight *lux* fusion plasmids used for the plant-free induction analysis with acetosyringone (above) were spotted on freshly sliced carrot disks in duplicates. Light production was easily detected with fusions to *virB* and *virE* and somewhat lower with fusions to *virD* (Fig. 4). While these promoters were turned on only in the presence of pTiC58, the unregulated *tet* promoter gave high levels of light production regardless of whether pTiC58 was present. In all other cases the amount of light produced was too low to be recorded by the photographic film, which is about 100-fold less sensitive than the luminometer (J. Shaw, personal communication). Regardless of this lower level of

TABLE 6. Effect of *virG* and *virA* on the expression of *virB* and *virE*

Plasmids in strain LBA4301	Copy no./cell			CAT activity (nmol/min per mg of protein)[a]			
				pUCD1550 (*virB*)		pUCD1553 (*virE*)	
	virG	*virA*	*vir-cat* fusion	With acetosyringone	Without acetosyringone	With acetosyringone	Without acetosyringone
pUCD1508, pUCD1545	0	0	4	9	8	35	33
pUCD1508, pUCD1516	4	0	4	27	29	531	351
pUCD1192, pUCD1545	20	0	4	191	184	1,494	1,304
pUCD1192, pUCD1516	24	0	4	247	267	2,844	2,343
pUCD1508, pUCD1541	0	4	4	9	6	45	42
pUCD1543, pUCD1545	0	20	4	10	10	39	38
pUCD1543, pUCD1541	0	24	4	11	8	32	41
pUCD1543, pUCD1516	4	20	4	2,488	28	2,459	497
pUCD1192, pUCD1541	20	4	4	3,598	199	5,764	1,338

[a] Determined 18 h after induction.

induction, the data demonstrate that *vir* gene induction with acetosyringone qualitatively resembles gene induction on wounded carrot tissue.

Identification of *vir* genes involved in the induction process. As pointed out above, the induction of cloned *vir* promoters by acetosyringone was dependent on the presence of pTiC58 in the bacterial host. To identify the functions of pTiC58 involved in induction, indicator plasmids carrying *virB-lux* (pUCD1187) or *virE-lux* (pUCD1194) fusions were introduced into strain LBA4301 harboring mutant Ti plasmids (Fig. 2). Transconjugants were assayed for light production in the presence and absence of acetosyringone under standard induction conditions. Induction was prevented by mutations in either *virA* (JK107) or *virG* (JK710), while five *virB* mutations (JK502, JK190, JK104, JK125, and JK210), two *virC* mutations (JK702 and JK503), three *virD* mutations (JK195, JK703, and JK504), and three *virE* mutations (JK516, JK505, and JK523) had no effect (data not shown). Therefore, the products of *virA* and *virG* are needed for the induction of *virB* and *virE*.

Copy number effect of *virA* and *virG* on *vir* gene induction. To further investigate the role of *virA* and *virG* in *vir* gene induction, the plasmids shown in Fig. 5 were combined in all possible ways, resulting in derivatives of LBA4301 with various copy numbers of *virA* and *virG* and an indicator plasmid for the promoter activity of either *virB* or *virE* (Table 6). Induction studies with acetosyringone showed that *virB* was expressed at a low basal level in the absence of both *virA* and *virG*. The presence of *virA* alone had no influence on this basal level, whether *virA* was present in 4, 20, or 24 copies per cell. In contrast, increasing the gene copy number of *virG* led to considerably elevated basal expression of *virB*. This elevation in *virB* expression was further increased 89-fold (4 copies of *virG*) or 18-fold (20 copies of *virG*) when acetosyringone and *virA* were also provided. Similar results were observed for the *virE* promoter, although for *virE* the basal level in the absence of both genes and the intermediate levels reached in the presence of different gene dosages of *virG* were considerably higher than in the case of the *virB* fusions.

Careful examination of Tables 3 and 4 indicates a similar copy number effect of *virG* in the regulation of other *vir* genes. Comparison of the two different *virD* fusion plasmids pUCD206-1 and pUCD206-7 (Table 3) shows a fivefold-higher uninduced basal level and a threefold-higher induced level for pUCD206-1, which contained an intact *virG* locus in addition to the *virD-cat* fusion. Considering the copy number of pUCD206-1, this strain harbored four extra copies of *virG* in addition to the one present on pTiC58. Similar effects can be seen in Table 4; the basal level of *virC* was increased in the presence of four copies of *virG*. While the different fusion points of *virD* to *cat* provide an alternative explanation in the first case, this does not apply to the *virC* fusions. Therefore, the gene dosage of *virG* affects not only *virB* and *virE*, but also *virC* and possibly *virD*.

DISCUSSION

We show here that the genetic organization and promoter activities of the pTiC58 *vir* region (Fig. 2) are structurally and functionally very similar to those of the *vir* region of octopine plasmids such as pTiA6. The genes *virA*, *virB*, *virC*, *virD*, *virE*, and *virG* were located in comparative studies involving three octopine plasmids with identical *vir* region restriction maps (as far as they have been published) and identical genetic organizations (13, 24, 31). Functional homologies between nopaline and octopine Ti plasmids were shown by cross-complementation of pTiC58 mutants with pTi15955 cosmid clones (Table 2). The exact endpoint of *virD* and the exact extent of *virB* have yet to be determined, and the suggestion that *virB* is an operon is based on analogies to the octopine Ti plasmids. It is supported by the fact that *lux* fusions to internal fragments not only of *virD* but also of *virB* do not show promoter activity.

Gene fusions to selected restriction fragments of the pTiC58 *vir* region revealed promoter activities at the 5′ ends of *virA*, *virB*, *virC*, *virD*, *virE*, and *virG*. All of these fusions occurred at well-defined sites within the respective ORFs, and it is therefore not possible that regulatory signals between promoter and ORF are missing. All upstream signals were probably also present, because bacterial promoters are in general considerably shorter than 800 bp. This was the minimum length of upstream sequences provided in all cases but *virE* (only 250 bp), in which the promoter was previously identified and confined to a 100-bp stretch (12). Of three additional promoter activities, only the one upstream of *tzs* corresponds to a known gene. Additional, weak promoter activities may have escaped detection because they were below the detection level of the Lux system.

Induction studies with acetosyringone resulted in a pattern similar to that of the octopine plasmid pTiA6 (31, 32) for the highly inducible loci *virB*, *virC*, *virD*, and *virE* as well as for the weakly inducible loci *virA* and *virG*. While two different *virA-lacZ* fusions of pTiA6 were induced 0.9- and 1.1-fold, respectively (31), *virA* of pTiC58 was induced 2.6-fold (Table 4). Similar results were obtained with subcloned fragments of both plasmids that were fused to *cat* (Table 4). The *lux* fusions of the pTiC58 gene also indicated an induction of *virA* (Fig. 3). Despite quantitative differences, both the luciferase and CAT assays led to the same conclusion: that *virA* is slightly induced in pTiC58 and is regulated similarly but not identically to its counterpart in pTiA6.

In the case of *virG*, the 13.6- and 12.7-fold induction of two different *virG-lacZ* fusions of the pTiA6 gene (31) is in sharp contrast to the 1.8-fold induction found in pTiC58. This discrepancy may reflect an actual difference in the regulation of the two genes, but one or more of the following explanations may at least partly account for it. First, the growth temperature for *A. tumefaciens* was 28°C in the work of Stachel et al. (31), while our work was performed at 25°C, the upper limit for reasonable luciferase activities. Second, the genetic background for the pTiA6 experiments was a recombination-proficient derivative of strain C58, which originally harbored the nopaline Ti plasmid pTiC58. In contrast, we monitored the gene expression of pTiC58 genes in a recombination-deficient derivative of strain Ach5 that was cured of its octopine plasmid. It was found recently (P. Zambryski, personal communication) that different *A. tumefaciens* strains show different protein patterns after induction with acetosyringone. Third, the differences may be system dependent.

To address these possibilities, we analyzed the *virG* genes of pTiC58 and pTiA6 under identical conditions (subcloned fragments fused to *cat*, Table 4). Both genes were weakly induced by both acetosyringone or a shift from pH 7.0 to 5.7 in the medium, and overall induction was slightly higher in the case of the pTiA6 gene (3.6-fold versus 1.8-fold). These data indicate that both *virG* alleles are regulated in a similar but not identical fashion and are consistent with comparative nucleotide sequence data of the *virG* promoter regions (Powell et al., in press), which show blocks of homology as well as pronounced rearrangements.

In a recent analysis, Northern blots (RNA blots) were used to study transcription of the pTiC58 *vir* region during cocultivation with tobacco cell suspensions (14). The number of 13 "transcribed regions" presented can be reconciled with only six *vir* loci presented in this study as follows. (i) Two of the transcribed regions can be mutated without loss of virulence and therefore do not represent true *vir* loci. This is the case for regions 3 (*tzs* [1]), and 12 (ORF5 of *virE* [12]). (ii) Two of the restriction fragments used in the Northern blots contained more than one *vir* locus. Due to their intermediate phenotype, regions 5 (*virA* and *virB*) and 9 (*virG* and *virC*) were counted as additional transcribed regions. (iii) As discussed by the authors, long mRNAs like the proposed 8.5-kb transcript of *virB* "would likely be very unstable," and we agree that *virB* is probably represented by regions 6 and 7 together. We therefore propose that the region studied (which does not include regions 1 and 2) contains only six *vir* loci.

The patterns of results obtained with *lux* and *cat* fusions were very similar (Table 3). The great advantage of the *lux* fusions is that they make it possible to study gene expression in vivo. The interaction between pathogen and host is not disrupted and can be monitored continuously. We used this unique feature to show that the in vitro *vir* gene induction by acetosyringone was qualitatively similar to induction on wounded plant tissue (Fig. 4). We showed also that there seemed to be no strict requirement for a plant or a minimal medium during *vir* gene induction, while the pH of the medium and a saturating inducer concentration were crucial for optimal induction (Table 5).

The functions of pTiC58 required for induction of *virB* and *virE* were identified as *virA* and *virG*. We also observed that increases in *virG* alone (without acetosyringone or *virA*) were sufficient to increase *vir* gene expression, implying that the VirG protein and not the VirA protein is the activator of transcription and that the VirG protein in its unactivated form has some enhancing activity. The VirA protein, in contrast, does not act by itself; *virA* showed no copy number effect and enhanced transcription only in the presence of acetosyringone and the *virG* product. The VirA protein thus seems to be the link between inducing compounds and the activator VirG. It may, for example, act itself as a modulator of VirG activity, or it may transport (and modify) acetosyringone, which then activates the VirG protein. Protein sequence homologies of VirA and VirG to other pairs of regulatory proteins (24, 26, 35; Powell et al., in press) seem to favor the first model, but further experiments are needed to directly demonstrate the proposed interaction of inducer, VirA protein, and VirG protein on a molecular level.

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LITERATURE CITED

1. **Akiyoshi, D. E., D. A. Regier, G. Jen, and M. P. Gordon.** 1985. Cloning and nucleotide sequence of the *tzs* gene from *Agrobacterium tumefaciens* strain T37. Nucleic Acids Res. **13:** 2773–2788.
2. **Close, T. J., P. M. Rogowsky, C. I. Kado, S. C. Winans, M. F. Yanofsky, and E. W. Nester.** 1987. Dual control of *Agrobacterium tumefaciens* Ti plasmid virulence genes. J. Bacteriol. **169:**5113–5118.
3. **Close, T. J., R. C. Tait, T. Hirooka, L. Kim, and C. I. Kado.** 1987. Molecular characterization of the *virC* genes of the Ti plasmids. J. Bacteriol. **169:**2336–2344.
4. **Close, T. J., R. C. Tait, and C. I. Kado.** 1985. Regulation of Ti plasmid virulence genes by a chromosomal locus of *Agrobacterium tumefaciens*. J. Bacteriol. **164:**774–781.
5. **Close, T. J., D. Zaitlin, and C. I. Kado.** 1984. Design and development of amplifiable broad-host-range cloning vectors: analysis of the *vir* region of *Agrobacterium tumefaciens* plasmid pTiC58. Plasmid **12:**111–118.
6. **Engebrecht, J., K. Nealson, and M. Silverman.** 1983. Bacterial bioluminescence: isolation and genetic analysis of functions from *Vibrio fischeri*. Cell **32:**773–781.
7. **Engler, G., A. Depicker, R. Maenhaut, R. Villarroel, M. Van Montagu, and J. Schell.** 1981. Physical mapping of DNA base sequence homologies between an octopine and a nopaline Ti plasmid of *Agrobacterium tumefaciens*. J. Mol. Biol. **152:**183–208.
8. **Gallie, D. R., M. Hagiya, and C. I. Kado.** 1985. Analysis of *Agrobacterium tumefaciens* plasmid pTiC58 replication region with a novel high-copy-number derivative. J. Bacteriol. **161:** 1034–1041.
9. **Gallie, D. R., S. Novak, and C. I. Kado.** 1985. Novel high- and low-copy stable cosmids for use in *Agrobacterium* and *Rhizobium*. Plasmid **14:**171–175.
10. **Hagiya, M., T. J. Close, R. C. Tait, and C. I. Kado.** 1985. Identification of pTiC58 plasmid-encoded proteins for virulence in *Agrobacterium tumefaciens*. Proc. Natl. Acad. Sci. USA **82:**2669–2673.
11. **Hirooka, T., and C. I. Kado.** 1986. Location of the right boundary of the virulence region on *Agrobacterium tumefaciens* plasmid pTiC58 and a host-specifying gene next to the boundary. J. Bacteriol. **168:**237–243.
12. **Hirooka, T., P. M. Rogowsky, and C. I. Kado.** 1987. Characterization of the *virE* locus of *Agrobacterium tumefaciens* plasmid pTiC58. J. Bacteriol. **169:**1529–1536.
13. **Hooykaas, P. J. J., M. Hofker, H. den Dulk-Ras, and R. A. Schilperoort.** 1984. A comparison of virulence determinants in an octopine Ti plasmid, a nopaline Ti plasmid, and an Ri plasmid by complementation analysis of *Agrobacterium tumefaciens* mutants. Plasmid **11:**195–205.
14. **Janssens, A., C. Genetello, M. Van Montagu, and P. Zambryski.** 1986. Plant cells induce transcription of the *Agrobacterium tumefaciens* nopaline pTiC58 virulence region. Plant Sci. **47:**185–193.
15. **Kado, C. I., M. G. Heskett, and R. A. Langley.** 1972. Studies on *Agrobacterium tumefaciens*: characterization of strains 1D135 and B6 and analysis of the bacterial chromosome, transfer RNA and ribosomes for tumor inducing ability. Physiol. Plant Pathol. **2:**47–57.
16. **Kado, C. I., and S. T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145:** 1365–1373.
17. **Kao, J. C., K. L. Perry, and C. I. Kado.** 1982. Indoleacetic acid complementation and its relation to host range specifying genes on the Ti plasmid of *Agrobacterium tumefaciens*. Mol. Gen. Genet. **188:**425–432.
18. **Klee, H. J., F. White, V. N. Iyer, M. P. Gordon, and E. W. Nester.** 1983. Mutational analysis of the virulence region of an *Agrobacterium tumefaciens* Ti plasmid. J. Bacteriol. **153:** 878–883.
19. **Langley, R.A., and C. I. Kado.** 1972. Studies on *Agrobacterium tumefaciens*. Conditions for mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and relationships of *A. tumefaciens* to crown-gall tumor induction. Mutat. Res. **14:**277–286.
20. **Leroux, B., M. F. Yanofsky, S. C. Winans, J. E. Ward, S. F. Ziegler, and E. W. Nester.** 1987. Characterization of the *virA* locus of *Agrobacterium tumefaciens*: a transcriptional regulator and host range determinant. EMBO J. **6:**849–856.
21. **Lin, B.-C., and C. I. Kado.** 1977. Studies on *Agrobacterium*

tumefaciens. VII. Avirulence induced by temperature and ethidium bromide. Can. J. Microbiol. **23**:1554–1561.

22. **Lundquist, R. C., T. J. Close, and C. I. Kado.** 1984. Genetic complementation of *Agrobacterium tumefaciens* Ti plasmid mutants in the virulence region. Mol. Gen. Genet. **193**:1–7.
23. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Melchers, L. S., D. V. Thompson, K. B. Idler, R. A. Schilperoort, and P. J. J. Hooykaas.** 1986. Nucleotide sequence of the virulence gene *virG* of the *Agrobacterium tumefaciens* octopine Ti plasmid: significant homology between *virG* and the regulatory genes *ompR*, *phoB* and *dye* of *E. coli*. Nucleic Acids Res. **14**:9933–9942.
25. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Nixon, B. T., C. W. Ronson, and F. M. Ausubel.** 1986. Two-component regulatory systems responsive to environmental stimuli share strongly conserved domains with the nitrogen assimilation regulatory genes *ntrB* and *ntrC*. Proc. Natl. Acad. Sci. USA **83**:7850–7854.
27. **Novick, R. P., R. C. Clowes, S. N. Cohen, R. Curtiss III, N. Datta, and S. Falkow.** 1976. Uniform nomenclature for bacterial plasmids: a proposal. Bacteriol. Rev. **40**:168–169.
28. **Shaw, J. J., and C. I. Kado.** 1986. Development of a *Vibrio* bioluminescence gene-set to monitor phytopathogenic bacteria during the ongoing disease process in a non-disruptive manner. Bio/technology **4**:560–564.
29. **Shaw, W. V.** 1975. Chloramphenicol acetyltransferase from chloramphenicol resistant bacteria. Methods Enzymol. **43**: 737–755.
30. **Stachel, S. E., E. Messens, M. Van Montagu, and P. Zambryski.** 1985. Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. Nature (London) **318**:624–629.
31. **Stachel, S. E., and E. W. Nester.** 1986. The genetic and transcriptional organization of the *vir* region of the A6 Ti plasmid of *Agrobacterium tumefaciens*. EMBO J. **5**:1445–1454.
32. **Stachel, S. E., E. W. Nester, and P. C. Zambryski.** 1986. A plant cell factor induces *Agrobacterium tumefaciens vir* gene expression. Proc. Natl. Acad. Sci. USA **83**:379–383.
33. **Stachel, S. E., and P. C. Zambryski.** 1986. *virA* and *virG* control the plant-induced activation of the T-DNA transfer process of *A. tumefaciens*. Cell **46**:325–333.
34. **Veluthambi, K., R. K. Jayaswal, and S. B. Gelvin.** 1987. Virulence genes *A*, *G*, and *D* mediate the double-stranded border cleavage of T-DNA from the *Agrobacterium* Ti plasmid. Proc. Natl. Acad. Sci. USA **84**:1881–1885.
35. **Winans, S. C., P. R. Ebert, S. E. Stachel, M. P. Gordon, and E. W. Nester.** 1986. A gene essential for *Agrobacterium* virulence is homologous to a family of positive regulatory loci. Proc. Natl. Acad. Sci. USA **83**:8278–8282.
36. **Yanofsky, M. F., S. G. Porter, C. Young, L. M. Albright, M. P. Gordon, and E. W. Nester.** 1986. The *virD* operon of *Agrobacterium tumefaciens* encodes a site-specific endonuclease. Cell **47**:471–477.