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Genetics

A plant cell factor induces *Agrobacterium tumefaciens vir* gene expression

(virulence gene expression/*vir* gene induction/plant-synthesized *vir*-inducing factor/bacterial–plant cell recognition)

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ABSTRACT **The virulence genes of *Agrobacterium* are required for this organism to genetically transform plant cells. We show that *vir* gene expression is specifically induced by a small (<1000 Da) diffusible plant cell metabolite present in limiting quantities in the exudates of a variety of plant cell cultures. Active plant cell metabolism is required for the synthesis of the *vir*-inducing factor, and the presence of bacteria does not stimulate this production. *vir*-inducing factor is (*i*) heat and cold stable; (*ii*) pH stable, although *vir* induction with the factor is sensitive above pH 6.0; and (*iii*) partially hydrophobic. Induction of *vir* gene expression was assayed by monitoring β-galactosidase activity in *Agrobacterium* strains that carry gene fusions between each of the *vir* loci and the *lacZ* gene of *Escherichia coli*. *vir*-inducing factor (partially purified on a C-18 column) induces both the expression in *Agrobacterium* of six distinct loci and the production of T-DNA circular molecules, which are thought to be involved in the transformation process. *vir*-inducing factor potentially represents the signal that *Agrobacterium* recognizes in nature as a plant cell susceptible to transformation.**

Many species of soil bacteria form specialized symbiotic and parasitic interactions with plant cells (1). To initiate an interaction a bacterium must first recognize its appropriate susceptible plant cell. This recognition can be used by the bacterium to activate bacterial genes whose products mediate the development and/or maintenance of the interaction. But how does the bacterium recognize a susceptible plant cell; what are the signals and how are they detected? Here we begin to characterize bacterial–plant cell recognition by studying the early interaction that occurs between the phytopathogen *Agrobacterium tumefaciens* and plant cells.

A. tumefaciens causes crown gall, a neoplastic disease of dicotyledonous plants, by transferring a specific segment of DNA, the T-DNA, from its large (200 kilobases) Ti plasmid to plant cells, where it becomes integrated into the plant nuclear genome and expressed (reviewed in ref. 2). Although the mechanism of T-DNA transfer and integration into the plant cell DNA has not been elucidated, the Ti plasmid genes required for these events have been identified. These genes are not contained within the T-DNA but are located within the ≈40-kilobase virulence (*vir*) region (3–5). Genetic analysis of this region has shown that it encodes six separate *vir* complementation groups (see Fig. 1; unpublished observations). The proteins encoded by these genes likely mediate specialized functions, and thus *vir* expression might be limited to when the bacterium is in the presence of plant cells susceptible to transformation. Such regulation could be accomplished if *Agrobacterium* has the ability to recognize a specific molecule(s) produced by these cells. Recognition could then trigger *vir* gene expression to initiate the steps of T-DNA transfer and integration.

In this paper, we show that *vir* gene expression in *Agrobacterium* is indeed specifically stimulated by dicotyledonous plants, demonstrating that *Agrobacterium* recognizes and responds to plant cells. We further show that *vir* induction is mediated by one or more low molecular weight partially hydrophobic molecules found in the exudates of metabolically active plant cells. We propose that this factor is the signal that *Agrobacterium* identifies in nature as a plant cell susceptible to transformation.

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MATERIALS AND METHODS

Plant Cell Cultures. *Nicotiana tabacum* cv. *xanthi* and cv. *W38* plants were used to prepare leaf disc cultures according to ref. 6. Root cultures of *Nicotiana glauca* (untransformed) and *N. tabacum* transformed with strain *Agrobacterium rhizogenes* A15834 (gift of J.-P. Hernalsteens) were grown in liquid Murashige and Skoog (MS) medium (7) (containing MS salts, 3% sucrose, 0.01% inositol, 0.0001% biotin, and 0.018% KH_2PO_4). Roots were grown in 100 ml of medium in 500-ml flasks at 26°C spun at 90 rpm; medium was changed weekly and the root mass was divided every 2 weeks to give an inoculum of ≈10 g. Callus suspension cell lines of *Vinca rosea* and *N. tabacum* were grown in 50 ml of MS medium supplemented with 2,4-dichlorophenoxyacetic acid (0.2 mg/ml) in 250-ml flasks at 26°C, shaking at 120 rpm. Every 7 days, 2 ml of the *N. tabacum* suspension culture was subcultured into 50 ml of fresh culture medium; under these growth conditions this culture saturates in 7 days. *Vinca rosea* was subcultured weekly at a 20% inoculation. Mesophyll protoplasts were prepared from *N. tabacum* leaves (8) and regenerated for 72 hr prior to use.

Preparation of Conditioned Medium. Forty-eight to 72 hr after subculture of *N. tabacum* roots into fresh medium, the conditioned medium was removed, filtered through 0.22-μm nitrocellulose, and stored at −20°C; this material was designated cmr (conditioned medium roots).

***vir* Expression Assays.** All bacterial strains and plasmids are described in Fig. 1 and Table 1. The plasmids used confer resistance to carbenicillin and kanamycin, and bacterial cultures were grown in YEB liquid medium (11) supplemented with each of these antibiotics at 100 μg/ml. Overnight cultures were pelleted and resuspended in ⅒th vol of MS plant medium. Material to be tested for *vir*-inducing activity was inoculated with bacteria at an absorbance of 0.1 OD units per ml at 600 nm (cm^{-1}). Specific units of β-galactosidase activity in *Agrobacterium* were determined as described (10) and are reported as units per bacterial cell.

RESULTS

***lac* Fusions as Probes for *vir* Expression.** *vir* expression was monitored by using *Agrobacterium* strains that carry gene

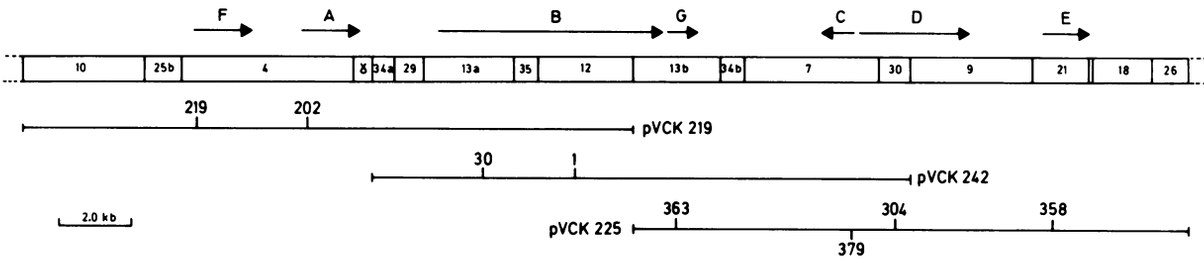

FIG. 1. pTiA6 *vir*::*lac* gene fusions. Map positions of *virA* through *virG*, and of the *pinF* locus (unpublished results) are indicated by arrows above the *Sal* I restriction map of the pTiA6 *vir* region. pVCK219, pVCK242, and pVCK225, three cosmid clones (9) that contain pTiA6 *vir* region sequences, are shown below as horizontal lines. These clones were mutagenized with the Tn*3*-*lacZ* transposon (10), Tn*3*-HoHo1, to generate plasmids carrying *vir*::*lacZ* gene fusions. The map positions within these clones of eight *vir*::*lacZ* fusions are indicated by vertical lines. The numbers designate the pSM(*vir*::*lac*) plasmids that carry the respective fusions. pSM219 and pSM202 were derived from pVCK219 and carry *pilF*::*lac* and *virA*::lac fusions, respectively; pSM30 and pSM1 were derived from pVCK242 and carry separate *virB*::*lac* fusions; and pSM363, pSM379, pSM304, and pSM358 were derived from pVCK225 and carry *lac* gene fusions to *virG*, *virC*, *virD*, and *virE*, respectively. [Each of the *vir*::*lacZ* insertions has been recombined onto pTiA6 to assess its effect on virulence (unpublished results). All these insertions produce an avirulent phenotype except for 219 and 379. The 379 insertion causes an attenuated virulence phenotype, while the 219 insertion does not affect virulence. The *F* locus in pTiA6 has been defined by its plant cell inducibility and is designated *pin* (plant-inducible locus) *F*. The transcriptional orientation of each of these fusions is leftward to rightward, except for the *virC*::*lac* fusion of pSM379, whose orientation is rightward to leftward.] The eight pSM(*vir*::*lac*) plasmids were placed into *A. tumefaciens* strain A348 to give eight A348(pSM*vir*::*lac*) strains. A348 contains the wild-type A6 Ti plasmid. kb, Kilobases.

fusions between the pTiA6 *vir* loci and *lacZ*. These fusions are described in detail elsewhere (ref. 10; unpublished observations) and are schematically shown, along with the genetic map of the pTiA6 *vir* region, in Fig. 1. In brief, they were constructed by randomly inserting the Tn*3*-*lacZ* transposon, Tn*3*-HoHo1 (10), into plasmid clones of the pTiA6 *vir* region (9). When this element inserts into a genetic locus such that the transcriptional orientation of the locus and the promoterless *Escherichia coli lac* operon carried by the element are in register, the production of the *lacZ* gene product, β-galactosidase, is placed under the control of the locus. The relative level of expression of a particular *vir* locus in *Agrobacterium* can thus be easily and quantitatively monitored by measuring the amount of β-galactosidase activity present in a cell carrying a *lac* fusion to the locus.

The *Agrobacterium vir*::*lac* strains provide a bioassay for the identification of conditions that stimulate *vir* expression.

Table 1. *vir* induction by protoplasts, cmr, and C-18 factor

Strain	Locus	β-galactosidase activity			
		MS⁻	Protoplast	cmr	C-18 factor
A348/pSM219	*pinF*	10.3	726.0	100.0	1289.0
A348/pSM202	*virA*	51.9	55.3	ND	61.7
A348/pSM30	*virB*	10.1	634.0	383.0	1124.0
A348/pSM1	*virB*	13.6	792.0	133.0	919.0
A348/pSM363	*virG*	72.6	941.0	277.0	1022.0
A348/pSM379	*virC*	3.9	58.2	5.6	105.0
A348/pSM304	*virD*	8.2	763.0	51.9	983.0
A348/pSM358	*virE*	56.7	2980.0	630.0	4034.0
A348/pSM102	*occ*	2.6	3.0	2.7	ND
A348/pSM102*	*occ*	84.4	72.9	61.8	ND

The β-galactosidase activity present in bacteria after incubation in MS medium, protoplast cultivation, cmr, and C-18 factor was determined for the eight A348(pSM*vir*::*lac*) strains shown in Fig. 1, and the *occ*::*lac* strain A348(pSM102). A348(pSM102) incubations were carried out in the absence or presence of octopine (100 μg/ml) (obtained from R. Jensen and from Sigma). Conditions of protoplast cocultivation were as reported (10) and β-galactosidase activity was assayed after 16-hr cocultivations. For the MS, cmr, and C-18 factor experiments, 1.5 ml of the respective material in Falcon tubes (17 × 100 mm) was inoculated with bacteria, and β-galactosidase activities were determined after a 12-hr incubation at 28°C and at 200 rpm and are expressed as activity units per bacterium. ND, not determined. *Incubation in the presence of octopine.

To control that such conditions are specific for *vir* induction, an *Agrobacterium* strain that carries *lacZ* fused to the pTiA6 octopine catabolism (*occ*) locus was also used (10). *occ* is genetically and functionally distinct from *vir*, and expression of *occ* (12), and not *vir* (10), is specifically induced by D-octopine [N^2-(1-carboxyethyl)-L-arginine]. Thus *occ* expression should not be affected by conditions that induce *vir*.

Plant Cells Induce *vir* Expression. The induction of *vir* gene expression by plant cells was initially established by using regenerating mesophyll protoplasts of *N. tabacum*. These cells are susceptible to high efficiency transformation by *Agrobacterium* (8, 13). During cocultivation of these cells with *Agrobacterium* carrying wild-type pTiA6 and *vir*::*lacZ* fusion plasmids, the levels of β-galactosidase activity in the bacteria greatly increased (up to 100-fold) for fusions to all the *vir* loci, with the exception of *virA*, whose expression is constitutive (unpublished results) (Table 1). This induction is *vir*-specific as cocultivation has no effect on *occ* expression; β-galactosidase activity in the *occ*::*lac* strain A348(pSM102) increased only if octopine was added to the cocultivation. We note that *vir* induction is, at least in part, an *Agrobacterium*-specific phenomenon: induction of β-galactosidase expression does not occur in *E. coli* that harbor our *vir*::*lacZ* plasmids (data not shown). Octopine induction of *occ* expression, however, occurs in both *Agrobacterium* and *E. coli* (10).

To further study *vir* induction, a more convenient source of plant material was desired. *vir* induction was seen to occur during cocultivation with several plant culture systems, including leaf discs of *N. xanthi* and *N. tabacum*, roots of *N. glauca* and *N. tabacum*, and suspension callus cell lines of *V. rosea* and *N. tabacum*. A particular *N. tabacum* callus suspension cell line, designated NT1, which consistently stimulates *vir* expression and can be directly transformed by *Agrobacterium* (G. An, personal communication), was chosen to characterize the phenomenon further.

Small Diffusible Factor Mediates Induction. While NT1 culture stimulates high levels of *vir* induction, the conditioned cell-free medium from this culture stimulates only low levels (from 0- to 6-fold) of induction (Table 2). Because bacteria both grow and exhibit normal levels of octopine-induced *occ* expression in this medium (Table 2), we inferred that the NT1 cells must be present for efficient *vir* induction to occur. We tested three possible explanations for this requirement: either physical contact between bacteria and plant cells is required; a *vir*-inducing factor(s) produced by plant cells is only

Table 2. NT1 plant metabolism affects the level of *vir* induction

Treatment	β-Galactosidase activity in A348(pSM⁻) strain				
	1	30	358	102/−oct	102/+oct
fm	14.0	10.2	59.4	2.55	84.4
2d	1007.0	767.0	1431.0	2.92	116.0
2d-5 ml	240.0	254.0	649.0	3.12	111.0
2d-boil	68.0	84.1	88.5	2.34	91.8
2d/cx	163.0	301.0	403.0	1.94	86.2
2d/fm	410.0	410.0	ND	3.02	47.0
2d/fm+cx	74.2	137.0	ND	2.63	76.1
6d	40.4	108.0	ND	2.58	82.6
6d/fm	379.0	473.0	ND	2.18	109.0
6d/fm+cx	35.6	24.2	ND	1.84	87.2
2dcm	41.7	68.8	106.0	2.79	99.9
2dcm-cc	27.6	32.4	74.4	ND	ND

Agrobacterium lac fusion strains A348(pSM1/*virB*), A348(pSM30/*virB*), A348(pSM358/*virE*), A348(pSM102/*occ*) were used to assess the *vir*-inducing activity of NT1 culture subjected to different treatments, and of conditioned medium from NT1 culture. Incubations were carried out in 2-ml volumes, unless otherwise indicated, for 16 hr at 28°C in 60-mm Petri dishes, and the units of β-galactosidase activity in the bacteria were then determined. A348(pSM102) incubations were carried out in the absence (−oct) and presence (+oct) of octopine (100 μg/ml). Materials tested were as follows: fm (fresh medium), MS medium supplemented with 2,4-dichlorophenoxyacetic acid (0.2 mg/ml); 2d (2-day NT1 culture), NT1 suspension culture 2 days after subculture; 2d-5 ml, 5 ml of the 2-day NT1 culture; 2d-boil, 2-day NT1 culture boiled for 15 min directly prior to cocultivation with bacteria; 2d/cx, 2-day NT1 culture simultaneously inoculated with bacteria and 20 μM cycloheximide; 2d/fm, the NT1 suspension cells of the 2-day culture were separated by centrifugation from culture medium, resuspended in an equivalent volume of fresh medium, and immediately inoculated with bacteria; 2d/fm+cx, as above, except the fresh medium contained 20 μM cycloheximide; 6d (6-day NT1 culture), NT1 suspension culture 6 days after subculture; 6d/fm, the NT1 suspension cells of the 6-day culture were separated by centrifugation from the culture medium and resuspended in fresh medium to a density corresponding to that of the 2-day culture; 6d/fm+cx, as in 6d/fm, except the fresh medium contained 20 μM cycloheximide; 2dcm (2-day conditioned medium), cell-free conditioned culture medium from 2-day NT1 culture; 2dcm-cc (2-day conditioned medium cocultivation), cell-free conditioned medium from a 16-hr cocultivation of 2-day NT1 culture and A348(pSM30) bacteria. ND, not determined.

Table 3. Effect of dialysis membrane on *vir* induction

Strain A348(pSM⁻)	Factor source		β-Galactosidase activity in bacteria	
			Outside	Inside
358	NT1 culture	(a)	1850	1362
		(b)	1721	—
		(c)	—	1412
30	NT1 culture	(a)	692	381
		(b)	515	—
		(c)	—	451
102	NT1 culture + octopine (100 μg/ml)	(a)	143	102
		(b)	129	—
		(c)	—	96
30	cmr	(a)	237	197
		(b)	220	—
		(c)	—	189

MS medium was separately inoculated with *Agrobacterium* strains A348(pSM358/*virE*), A348(PSM30/*virB*), and A348(pSM102/*occ*). Each inoculated medium (5 ml) was enclosed in Spectrapor 6 dialysis membrane (VWR), with an exclusion limit of 1000 Da (Stokes radius). In parallel experiments, 5 ml of uninoculated MS medium was enclosed in the membrane. The dialysis bags were incubated in 250-ml flasks with 50 ml of 2-day NT1 culture inoculated with bacteria. Dialysis bags were also incubated in uninoculated NT1 culture. In a parallel set of experiments, cmr was used in place of NT1 culture. Three experiments were set up for each bacterial strain: (a) bacteria both inside and outside the dialysis bag; (b) bacteria only outside the dialysis bag; and (c) bacteria only inside the dialysis bag. After 16 hr of incubation at 28°C and at 120 rpm, the β-galactosidase activity units of each population of bacteria was determined. In the A348(pSM102) experiments, octopine (100 μg/ml) was added to the NT1 culture. The size of octopine is 246 Da.

produced when bacteria are present; or the inducing factor is constitutively produced by the NT1 cells and is present in their exudate in quantities limiting for *vir* induction.

First, we determined whether physically blocking plant–bacterial contact during cocultivation affects *vir* induction (Table 3). Bacteria were enclosed within a dialysis bag prior to incubation with NT1 culture. Parallel incubations contained bacteria in contact with NT1 cells, or bacteria both inside and outside a dialysis bag in an NT1 culture. After 16 hr, the level of induced β-galactosidase activity in the bacteria inside the bag was approximately equivalent to that in the bacteria outside the bag. Also, the presence or absence of bacteria outside the dialysis bag had no significant effect on the induction levels of bacteria inside the bag.

The above data demonstrate that the stimulation of *vir* expression by NT1 cells does not require plant–bacterial contact and is mediated by a soluble factor. We estimate the upper limit of its size to be 1000 Da, the exclusion limit of the dialysis membrane used in these experiments.

Efficient Induction Requires Plant Metabolism. In our standard NT1 induction assay, a fixed number of bacteria are cocultivated for 16 hr with 2 ml of suspension culture in 60-cm Petri plates. Under these conditions, a 2-day subculture induced β-galactosidase activity 70-fold in the *virB*::*lac* strain A348(pSM1). Lower levels of induction were obtained in parallel cocultivations in which NT1 cell metabolism was restricted. For example, induction was 12-fold when cycloheximide, an inhibitor of eukaryotic translation, was added, and 5-fold when the NT1 culture was boiled prior to cocultivation. Restricted culture aeration also reduced induction: when the volume of 2-day culture was increased from 2 ml to 5 ml in the cocultivation, A348(pSM1) induction decreased to 17-fold (Table 2). Also, in large-volume cocultivations, efficient *vir* induction occurs in shaking, but not stationary, NT1 suspension cultures (data not shown).

The level of *vir* induction is also affected by the age of the NT1 culture (Table 2). A 6-day culture induced A348(pSM1) only 3-fold; however, when the NT1 cells from this culture were subcultured in fresh medium prior to cocultivation, 27-fold induction was obtained. Furthermore, cycloheximide blocked this effect. We note that the levels of octopine-induced *occ* expression were not significantly affected by any of these treatments.

The above results demonstrate that continual NT1 cell metabolism during cocultivation is required to produce *vir*-inducing factor in sufficient quantity for efficient *vir* induction. This production is likely constitutive and not a response to the presence of *Agrobacterium*, as conditioned medium from a cocultivation induced *vir* no better than conditioned medium from an NT1 culture that had never seen bacteria (Table 2). Thus, the factor is likely a plant cell metabolite.

Physical Properties of Factor. To characterize *vir*-inducing molecules, it is necessary to have a culture system that produces factor in sufficient quantities to allow detection by bioassay in the absence of plant cells. Conditioned media from *N. glauca* and *N. tabacum* root cultures, designated cmr (conditioned medium roots), were found to contain detectable activity in the absence of root cells.

Several experiments were carried out to characterize the *N. tabacum* cmr factor. Essentially identical results have also been obtained with the *N. glauca* cmr (data not shown). The cmr factor is soluble and small, since it passes through

dialysis membrane with an exclusion limit of 1000 Da (Table 3). The cmr factor is heat stable and cold stable: when cmr was either boiled for 15 min or frozen for several months at −20°C prior to bioassay, its original inducing activity was fully retained. The cmr factor is pH stable: when cmr was adjusted to pH 1.0 or pH 11.0, incubated at 28°C for 15 min, and readjusted back to pH 5.5, *vir*-inducing activity was fully retained. Conversely, *vir* induction by cmr is pH sensitive: induction of the *virB*::*lac* strain A348(pSM30) by cmr that had been pH-adjusted to cover a range of 5.0–7.0, dropped from 40-fold at pH 5.5 to 0-fold at pH 6.0 and above (Fig. 2). This effect is *vir* specific, because the pH of cmr had no effect on octopine induction of *occ*. The sensitivity of *vir* induction to pH might result from an alteration in conformation of the inducing molecule, or of a bacterial component of *vir* induction.

Fig. 3 presents the induction kinetics of the β-galactosidase activity in the *virD*::*lac* strain A348(pSM304) during incubation with *N. tabacum* root culture, and with cmr obtained from this culture. At 10 hr, the level of induction stimulated by cmr was ⅙th that stimulated by root culture. cmr did not further stimulate induction after this time, whereas induction continued to increase steadily in the presence of roots. Thus, the inducing activity in cmr is limiting and the presence of roots is required for high induction levels. This limitation can be overcome if fresh cmr is added at various times to the bacteria/cmr culture. After 30 hr of incubation, the level of *vir* induction of twice-refreshed bacteria was 46% that of bacteria cocultivated with roots.

Results of similar experiments using other *lac* fusion strains indicate that different *vir* loci are differentially induced by cmr (Table 1). The induction of the *virB*::*lac* strain A348(pSM30) by plant cell culture and by nonrefreshed cmr was 63-fold and 38-fold, respectively. Conversely, *virC*::*lac* strains that are induced ≈15-fold by plant cell culture exhibited <2-fold stimulation by cmr. The inducibility by cmr of the other *vir* loci falls between that of *virB* and of *virC*.

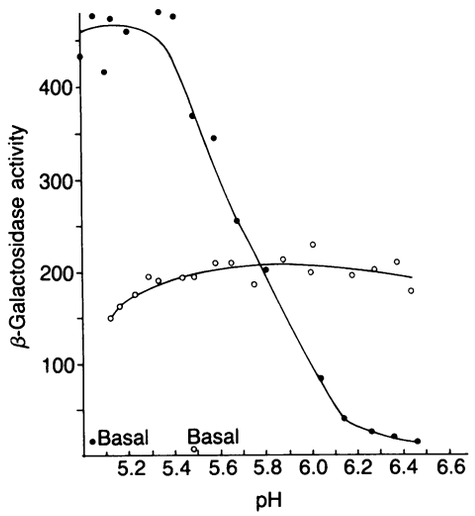

FIG. 2. Effect of pH on *vir* induction by cmr. *Agrobacterium virB*::*lac* strain A348(pSM30) (●) or *occ*::*lac* strain A348(pSM102) (○) were incubated in 2-ml samples of cmr, which were adjusted with sodium phosphate to a final concentration of 12.5 mM to cover a pH range between 5.0 and 7.0. The starting pH of the cmr was 5.5. For the *occ*::*lac* incubations, octopine (100 μg/ml) was included in the medium. All incubations were for 8 hr at 28°C and shaking at 200 rpm. The pH of each cmr sample was determined directly prior to assay of the β-galactosidase activity of the bacteria in the sample. The basal β-galactosidase activities of A348(pSM30) and A348(pSM102) after growth in MS medium/12.5 mM sodium phosphate, pH 5.5, were 10.1 and 2.69 units, respectively.

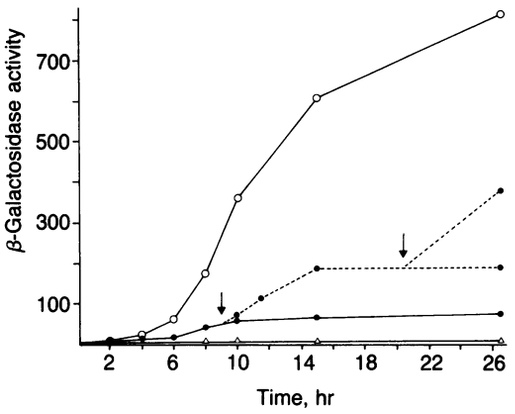

FIG. 3. Plant factor is limiting for *vir* induction. *Agrobacterium virD*::*lac* strain A348(pSM304) was separately inoculated into 50 ml of *N. tabacum rhizogenes*-transformed root culture (○), the conditioned-medium derived from this culture (cmr) (●), and unconditioned MS medium (△). Incubations were carried out in 250-ml flasks at 28°C and shaking at 120 rpm. At 2-hr intervals, 2-ml aliquots were removed and β-galactosidase activity of the bacteria was determined. At 9 hr and 20 hr, the cmr culture was refreshed with an equivalent volume of fresh cmr as indicated by arrows.

Thus, the cmr factor might induce the expression of some, but not all, *vir* loci. Conversely, this factor might induce all of *vir* but greater amounts are required to stimulate *virC* than *virB*.

These questions were resolved by using concentrated cmr factor. To this end, different gel chromatography matrices were tested for their ability to retain the cmr factor. Silica C-18 was found to retain the *vir*-inducing activity quantitatively, indicating that it has some hydrophobic character. cmr (150 ml) was passed through a rapid sample Sep-Pak C-18 cartridge (Waters Associates). The cartridge was step-eluted with 10 ml each of H_2O, and 20%, 40%, and 90% CH_3OH/H_2O (vol/vol). After lyophilization, each elution sample was resuspended in 1.5 ml of MS medium containing 12.5 mM sodium phosphate (pH 5.5) (phosphate was added to aid bacterial growth). These four samples, along with the cartridge flow-through material, were bioassayed for *vir*-inducing activity. Activity was only found in the 40% CH_3OH-eluted fraction; this material, designated C-18 factor, was stored at −20°C.

Biological Activity of C-18 Factor. Two tests confirmed the biological activity of the C-18-concentrated factor. First, the inducibility of several *vir*::*lac* strains with factor (corresponding to a 100-fold concentration of the original cmr) was determined. Induction of all the inducible *vir* loci was obtained (Table 1), and the levels of induction were equivalent to, or higher than, those obtained with regenerating protoplasts or roots. Even the *virC*::*lac* strain A348(pSM379), which was not significantly induced by cmr, was induced 35-fold in the presence of the C-18 factor, suggesting that *virC* might require higher levels of factor than other *vir* loci for its induction.

Second, we tested whether the concentrated factor could induce T-DNA-associated molecular events, which have been identified to occur within *Agrobacterium* during cocultivation with mesophyll protoplasts. In the Ti plasmid, the T-DNA is defined and bounded by identical 25-base-pair direct repeats; only DNA between these T-DNA borders is seen to be transferred to the plant genome (reviewed in ref. 14). During cocultivation with plant cells, independent T-DNA circles are formed in *Agrobacterium* by a specific recombination between the 25-base-pair sequences at the ends of the T-DNA (15).

We assayed whether C-18 factor could induce T-DNA circles in *Agrobacterium* carrying Ti plasmid pGV3850 (16).

The T-DNA of pGV3850 consists of the cloning vehicle pBR322 flanked by the left and right T-DNA border regions, and T-DNA circular intermediates can be isolated by transforming *E. coli* with DNA isolated from pGV3850 and selecting for the carbenicillin-resistance marker of the pBR322 portion of the pGV3850 T-DNA (15). Total DNA prepared from five independent incubations (two experiments for 12 hr and three experiments for 24 hr) of *Agrobacterium* containing pGV3850 in C-18 factor gave 20 and 33, and 44, 70, and 86 T-DNA circular intermediates per μg of DNA, respectively. In comparison, total *Agrobacterium* DNA prepared after three independent cocultivations (for 48 hr) of *Agrobacterium* containing pGV3850 with regenerating protoplasts gave 36, 40, and 48 T-DNA circular intermediates per μg of DNA. Uninduced *Agrobacterium* containing pGV3850 never produces these intermediates. The transformation efficiency in all these experiments was equivalent and determined to be 6×10^6 transformants per μg with supercoiled pBR322 DNA. These data confirm that concentrated cmr factor induces biologically significant events, associated with plant cell transformation, in *Agrobacterium*.

DISCUSSION

The phytopathogen *A. tumefaciens* is able to genetically transform plant cells and this process is mediated, in part, by the gene products of the Ti plasmid *vir* genes. We show that the expression of the *vir* genes in *Agrobacterium* is induced by a variety of dicotyledonous plants and by several types of plant cells. We have investigated this phenomenon with regard to the production and properties of the plant cell factor that mediates *vir* induction.

vir-inducing activity is produced in quantities limiting for *vir* induction, and active plant cell metabolism is required for this production. Also, production of inducing activity is not significantly affected by the presence of bacteria, suggesting that it is not regulated by *Agrobacterium*. The *vir*-inducing activity is a diffusible molecule(s) present in plant cell exudates. Semipurified and concentrated exudate induces the expression of each of the inducible *vir* loci to levels equivalent to, or greater than, those induced by cocultivation.

Our results indicate that plant cell induction of the *vir* gene expression in *Agrobacterium* is a *vir*- and *Agrobacterium*-specific phenomenon that is mediated by an inducing factor composed of one or more small, stable plant cell metabolites. These findings are not in agreement with a recent report that a heat-labile proteinaceous factor of >7000 Da induces *vir* expression both in *E. coli* and *Agrobacterium* (17); a single *vir*::*lac* gene fusion was used to assess *vir* induction, which is reported as qualitative changes in β-galactosidase activity. In the present work *vir* expression is quantitatively monitored by using gene fusions between each of the *vir* loci and *lacZ*, and nonspecific metabolic effects are controlled for by using a *lacZ* fusion to the metabolic *occ* locus. We have also directly monitored *vir* induction by measuring *vir*-encoded RNA in uninduced and factor-induced bacteria (unpublished results) and by measuring a functional product of *vir* induction, T-DNA intermediates.

How the *vir* factor mediates induction is not known. It might act at the surface of the bacterial cell to trigger a secondary messenger system, or it might act directly within the cell; the fact that the factor is small and diffusible suggests that it might enter the bacterium. The pTiA6 *vir* region is seen to function as a single regulon whose induction is attenuated in *virA*, and does not occur in *virG*, mutant bacteria (unpublished observations). Conceivably, the *virA* and *virG* gene products might function in *Agrobacterium* as the receptor and the effector molecules for the *vir*-inducing factor.

It is not known whether the *vir* gene loci are transferred to and expressed in the plant cell during the transformation process. These sequences are not found integrated in the plant genome, and our data demonstrate that all the *vir* loci are expressed in a regulated fashion in the bacterium. Thus, if *vir*-encoded proteins function in the plant cell during transformation, they are likely first synthesized in the bacterium and then transferred to the plant cell, perhaps as part of a T-DNA–protein complex.

In nature, only wounded plant cells are susceptible to transformation by *Agrobacterium* (18). A primary step in transformation should thus be the detection and recognition by the bacterium of such plant cells. The *vir*-inducing factor that we have begun to characterize could be the signal that *Agrobacterium* recognizes in nature as a wounded plant cell. We note that all the plant cultures determined to produce this factor contain, to some degree, mechanically damaged cells.

The present analysis offers insight into how a bacterium recognizes a plant cell. This work should provide a basis for the study of other bacterial–plant interactions, and it may have practical application for the promotion of useful, and the prevention of harmful, bacterial–plant cell interactions.

We have recently identified the cmr *vir*-inducing activity (19) to be composed of at least two derivatives of acetophenone that each separately fully activates the *vir* regulon.

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