JOURNAL OF BACTERIOLOGY, July 1989, p. 3696–3703
0021-9193/89/073696-08$02.00/0
Copyright © 1989, American Society for Microbiology
Vol. 171, No. 7

Opines Stimulate Induction of the *vir* Genes of the *Agrobacterium tumefaciens* Ti Plasmid

K. VELUTHAMBI,[1†] M. KRISHNAN,[1] JEAN H. GOULD,[2] ROBERTA H. SMITH,[2] AND STANTON B. GELVIN[1*]

Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907,[1] and Department of Soil and Crop Sciences, Texas A&M University, College Station, Texas 77843[2]

Received 9 February 1989/Accepted 5 April 1989

Upon incubation of *Agrobacterium tumefaciens* A348 with acetosyringone, the *vir* genes encoded by the Ti (tumor-inducing) plasmid are induced. The addition of certain opines, including octopine, nopaline, leucinopine, and succinamopine, enhanced this induction 2- to 10-fold. The compounds mannopine, acetopine, arginine, pyruvate, and leucine did not stimulate the induction of the *vir* genes to such an extent. The enhancement of *vir* gene induction by opines depended on acetosyringone and the genes *virA* and *virG*. Opines stimulated the activity of the *vir* genes, the double-stranded cleavage of the T (transferred)-DNA at the border repeat sequences, and the production of T-strands by the bacterium. The transformation efficiency of cotton shoot tips was markedly increased by the addition of acetosyringone and nopaline at the time of infection.

Downloaded from http://jb.asm.org/ on September 21, 2018 by guest

Phenolic compounds secreted by wounded plant cells can induce the activity of certain genes encoded by the Ti (tumor-inducing) plasmid of *Agrobacterium tumefaciens* (3, 37, 39). These inducible genes include the *vir* genes, which function in the process of T (transferred)-DNA excision and transfer, and the gene *pinF*, of unknown function (38). This induction process is mediated by the genes *virA* and *virG* (42). *virA*, a constitutively expressed gene, encodes an inner membrane-localized protein (22) that most likely functions as the receptor (42, 51) for such phenolic inducers as acetosyringone (37). The *virG* locus, which is expressed at a low basal level, is subsequently induced to higher levels of expression (38). This gene presumably encodes a transcriptional activator that induces its own expression and the expression of the genes *virB*, *virC*, *virD*, *virE*, and *pinF* (27, 42, 51). The *virD* locus encodes a T-DNA border-specific endonuclease (1, 17, 41, 49, 52, 53) that nicks the T-DNA border repeat sequences on the bottom strand, generating single-stranded T-DNA molecules called T-strands (1, 40, 48). T-strands can be coated with a *virE*-encoded single-stranded-DNA-binding protein (6, 8, 11, 15) and have been hypothesized to be the form of the T-DNA transferred to the plant cell (43). The *virD* endonuclease also generates double-stranded scissions at the T-DNA borders (17, 47, 48). The regulation of the genes *virC* and *virD* by the chromosomal locus *ros* has also been described (9, 10, 44).

Following insertion into plant nuclear DNA, T-DNA genes are transcribed and direct the synthesis of enzymes involved in the production of phytohormones and unusual low-molecular-weight compounds termed opines (for reviews, see references 2, 16, and 31). The phytohormones stimulate the uncontrolled proliferation of plant cells, resulting in tumors called crown gall tumors. Opines, which are crown gall tumor-specific compounds, are secreted into the rhizosphere where they can be used as an energy source by the inciting bacterium to the exclusion of most other soil microorganisms. It has been hypothesized that *Agrobacterium* species thereby creates for itself an ecological niche by "genetically engineering" the plants to synthesize compounds, the opines, that it can utilize (45, 46).

We are interested in the relationship, if any, between opines and *vir* gene induction. We initially hypothesized that opines may inhibit the induction of these genes by acetosyringone. The rationale was that the bacterium would not induce the T-DNA transfer machinery if it sensed that a tumor, recognized by the presence of opines, had already been formed. The results presented below suggest that many opines do affect the acetosyringone induction of the *vir* genes. The effect, however, is to enhance rather than repress this induction. The opines octopine, nopaline, leucinopine, and succinamopine enhance the acetosyringone induction of the *vir* genes, the formation of T-strands by the bacterium, and the transformation efficiency of certain dicotyledonous plants.

MATERIALS AND METHODS

Bacterial strains and growth conditions. We used *A. tumefaciens* A348 (21) harboring the Ti plasmid pTiA6 and derivatives for these studies. Stachel and Nester (38) generated strains harboring the transposon Tn*3*-HoHo1 in the virulence region. We used strains mutated in *virA* (A348mx211), *virB* (A348mx243), *virC* (A348mx365), *virD* (A348mx304), *virE* (A348mx358), *virG* (A348mx321), and *pinF* (A348mx219). Strain A136(pSM405) harbors the cosmid pSM405 (*virB*::Tn*3*-HoHo1 [42]) in the Ti plasmid-less *A. tumefaciens* A136. The cosmid pSM405 contains wild-type copies of *pinF*, *virA*, *virG*, and *virC* and contains a Tn*3*-HoHo1 insertion in *virB* (42). We mobilized this cosmid from *Escherichia coli* HB101 into *A. tumefaciens* A136 (which lacks a Ti plasmid) by triparental mating with the helper plasmid pRK2013 (12). pRGUS2 (46a), containing a β-glucuronidase (GUS) gene cloned between the CaMV 35S promoter and the nopaline synthase polyadenylation signal, was similarly mobilized into *A. tumefaciens* LBA4404 (32). *A. tumefaciens* strains were grown at 30°C on either AB minimal medium supplemented with 0.5% sucrose or YEP medium (23). *E. coli* strains were grown at 37°C on LB medium (26). The antibiotic concentrations used for *A. tumefaciens* were (micrograms per milliliter): rifampin, 10; carbenicillin, 100; kanamycin, 100. The antibiotic concentra-

* Corresponding author.
† Present address: Department of Biotechnology, School of Biological Sciences, Madurai Kamaraj University, Madurai 625021, India.

TABLE 1. Induction of *VIR* and *pinF* loci by acetosyringone and octopine[a]

Locus	β-Galactosidase (U)				Fold induction	
	K3	K3 + OCT	K3 + AS	K3 + AS + OCT	AS/K3	AS + OCT/AS
pinF	7.4 ± 1.1	10.4 ± 1.7	148.0 ± 27.6	332.0 ± 21.5	20.0	2.3
virA	18.3 ± 0.54	18.9 ± 1.1	18.1 ± 0.4	20.0 ± 1.5	1.0	1.1
virB	0.6 ± 0.2	0.6 ± 0.16	13.0 ± 3.3	70.1 ± 8.1	22.0	5.4
virG	41.0 ± 0.89	54.1 ± 1.2	38.2 ± 2.4	54.1 ± 1.15	0.9	1.4[b]
virC	2.0 ± 0.4	2.2 ± 0.4	10.0 ± 0.6	25.5 ± 0.6	5.0	2.5
virD	1.4 ± 0.17	1.8 ± 0.2	13.8 ± 0.9	44.3 ± 8.2	10.0	3.2
virE	12.7 ± 1.1	14.7 ± 0.8	26.6 ± 5.3	133.0 ± 24.6	2.1	5.0

[a] Acetosyringone (AS) was used as 30 μM; octopine (OCT) was used at 10 mM. All values are means of four replicate experiments.
[b] Induction by octopine was acetosyringone independent.

tions used for *E. coli* were (micrograms per milliliter): ampicillin, 100; kanamycin, 50.

Enzymes and reagents. Restriction endonucleases were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.) and used according to the recommendations of the supplier. [α-^{32}P]dCTP (800 Ci/mmol) and the nick translation kit were purchased from Amersham Corp. (Arlington Heights, Ill.). Acetosyringone was purchased from Aldrich Chemical Co., Inc. (Milwaukee, Wis.). Octopine, nopaline, and mannopine were purchased from Sigma Chemical Co. (St. Louis, Mo.). [^{14}C]nopaline, chemically synthesized from α-ketoglutarate and [^{14}C]arginine (270 mCi/mmol; ICN Pharmaceuticals Inc., Irvine, Calif.) as previously described (19), and leucinopine, succinamopine, and acetopine, synthesized chemically, were a kind gift from W. Scott Chilton.

Induction of *vir* genes. The *vir* genes were induced in K3 medium (30) supplemented with 5 mM MES (morpholineethanesulfonic acid) buffer (pH 5.6) with either acetosyringone at the indicated concentrations or regenerating tobacco protoplasts as previously described (47). K3 medium has the following composition (milligrams per liter): $NaH_2PO_4 \cdot H_2O$, 150; $Ca(H_2PO_4)_2 \cdot H_2O$, 46.1; $CaCl_2 \cdot 2H_2O$, 900; KNO_3, 2,500; NH_4NO_3, 250; $(NH_4)_2SO_4$, 134; $MgSO_4 \cdot 7H_2O$, 250; $FeSO_4 \cdot 7H_2O$, 27.8; disodium EDTA, 37.3; $MnSO_4 \cdot H_2O$, 7.6; H_3BO_3, 3.0; $ZnSO_4 \cdot 7H_2O$, 2.0; $Na_2MoO_4 \cdot 2H_2O$, 0.25; $CuSO_4$, 0.025; $CoCl_2 \cdot 6H_2O$, 0.025; KI, 0.75; nicotinic acid, 1.0; thiamine hydrochloride, 10.0; pyridoxine hydrochloride, 1.0; myoinositol, 100.0; α-naphthaleneacetic acid, 2.0; 2,4-dichlorophenoxyacetic acid, 0.1; benzylamino purine, 0.2; xylose, 250; and sucrose, 0.4 M (adjusted to pH 5.6 with NaOH). K3 medium has a low buffering capacity, and we noted that the pH of unsupplemented medium would often drop rapidly during the incubation periods. We monitored *vir* gene activity by measuring β-galactosidase activity in strains harboring Tn*3*-HoHo1 insertions in the *vir* genes as previously described (47).

Analysis of DNA. We extracted DNA from *A. tumefaciens* and analyzed it for the presence of T-strands and double-stranded cleavage at the T-DNA borders as previously described (48).

Nopaline uptake experiments. *A. tumefaciens* A348 was grown overnight in YEP medium, and 0.2 ml of the culture was inoculated into 25 ml of AB minimal medium and grown to a Klett reading of approximately 100. The cells were harvested and suspended in K3 medium supplemented with 5 mM MES (pH 5.6) at a concentration of 2×10^8 cells per ml. A 1-ml sample of the bacterial suspension was incubated at 25°C for various lengths of time in the presence of unlabeled nopaline (1 μM) plus [^{14}C]nopaline (50,000 cpm, 270 mCi/mmol). When appropriate, the bacteria were incubated with sodium azide (10 mM) for 1 h prior to the addition of nopaline. At the end of the incubation period, the sample was filtered through a nitrocellulose membrane (0.2-μm pore size; GSTF; Millipore Corp., Bedford, Mass.) and the cells were washed twice with 3 ml of K3 medium. The radiation on the filter was counted in a liquid scintillation counter.

Transformation of cotton. We infected shoot apex explants of cotton (*Gossypium hirsutum* var. Coker 312) with *A. tumefaciens* LBA4404(pRGUS2) as described by Ulian et al. (46a). After 2 days of infection, we transferred the shoot tips to medium containing 500 μg of carbenicillin per ml for 1 week and then to medium containing 7.5 μg of kanamycin per ml and 500 μg of carbenicillin per ml. We subsequently transferred the shoots weekly to fresh medium containing 500 μg of carbenicillin per ml. When used, nopaline or acetosyringone or both were added to the cut surface of the shoot apices in a drop of dimethyl sulfoxide at the time of inoculation. Tissue from the newest leaves (2 to 5 mg) was used to measure GUS activity fluorimetrically with 4-methyl umbelliferyl glucuronide as the substrate (18).

RESULTS

Characterization of acetosyringone induction. To characterize the induction of Ti plasmid genes by acetosyringone,

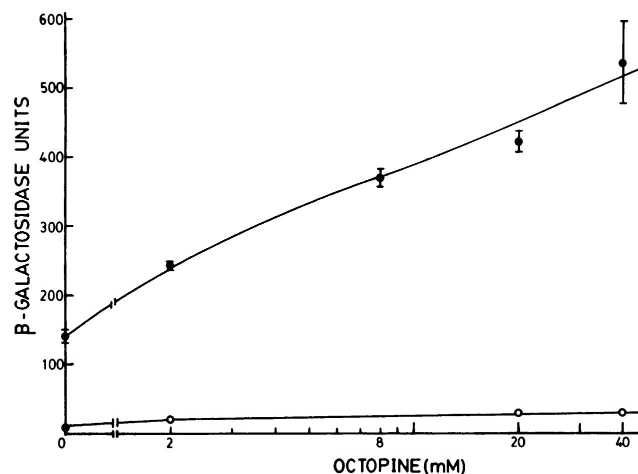

FIG. 1. Effect of octopine concentration on the enhancement of *pinF* induction by acetosyringone. *A. tumefaciens* A348mx219 harboring a Tn*3*-HoHo1 insertion in the gene *pinF* was grown to a Klett reading of 100 in AB medium plus 0.5% glucose containing 100 μg of carbenicillin per ml, centrifuged, and suspended in supplemented K3 medium containing (●) or lacking (○) 30 μM acetosyringone and various concentrations of octopine. After 24 h of incubation at 20°C, the cells were harvested and assayed for β-galactosidase activity.

Downloaded from http://jb.asm.org/ on September 21, 2018 by guest

TABLE 2. Effect of opines and related compounds on *vir* gene induction[a]

Treatment	*virB*			*pinF*		
	−AS	+AS	Fold increase over AS alone	−AS	+AS	Fold increase over AS alone
K3, Control	0.9 ± 0.5	5.0 ± 0.25		9.8 ± 0.9	126 ± 8.6	
K3 + octopine	1.5 ± 0.48	58.0 ± 3.8	11.6	17.4 ± 1.8	365 ± 18.3	2.9
K3 + nopaline	2.6 ± 1.3	189.0 ± 54.7	37.8	40.0 ± 2.7	808 ± 50.0	6.4
K3 + arginine	1.7 ± 0.29	27.0 ± 2.3	5.4	6.1 ± 1.0	167 ± 4.05	1.3
K3 + leucine	0.9 ± 0.25	13.4 ± 2.8	2.7	5.3 ± 1.7	161 ± 14.7	1.3
K3 + pyruvate	1.8 ± 0.6	7.6 ± 1.1	1.5	14.4 ± 1.9	109 ± 3.7	0.9

[a] Acetosyringone (AS) was used at 30 μM. Opines and other compounds were used at 10 mM. All values are means of four replicate experiments.

we used *A. tumefaciens* A348mx219. This strain contains the *lacZ* fusion transposon Tn*3*-HoHo1 (36) inserted into the gene *pinF* (38). Although *pinF* is not involved in virulence, its transcription is inducible by acetosyringone and cocultivation with plant protoplasts in a manner similar to that of many of the other *vir* genes (38), and β-galactosidase activity was high relative to that seen following the induction of other *vir* genes (Table 1). *pinF* is therefore a useful reporter of acetosyringone inducibility. The induction of *pinF* can be observed at acetosyringone concentrations as low as 3 μM and is maximal at 30 μM. These concentrations were therefore used in the following experiments for limiting (3 μM) or saturating (30 μM) induction conditions. Although a small amount of induction of *pinF* can be observed as early as 2 to 4 h following incubation with acetosyringone, the increase in *pinF* activity was most marked after 6 h. We chose 24 h as a convenient induction time to perform most of the experiments described below. The kinetics of induction and the concentrations of acetosyringone necessary for limiting and maximal induction were similar to those observed by Stachel et al. (37, 41).

Effect of opines on *pinF* and *vir* gene induction. We incubated *A. tumefaciens* A348mx219 with 30 μM acetosyringone for 24 h in K3 medium in the presence or absence of 10 mM octopine. The expression of *pinF* (as determined by β-galactosidase activity) was 2.3-fold greater in the presence of octopine than in its absence (Table 1). This induction by octopine depended on the presence of acetosyringone. Octopine alone did not induce *pinF* (Table 1). The synergistic effect of octopine on the acetosyringone induction of *pinF* was approximately linear in the range investigated (2 to 40 mM) (Fig. 1).

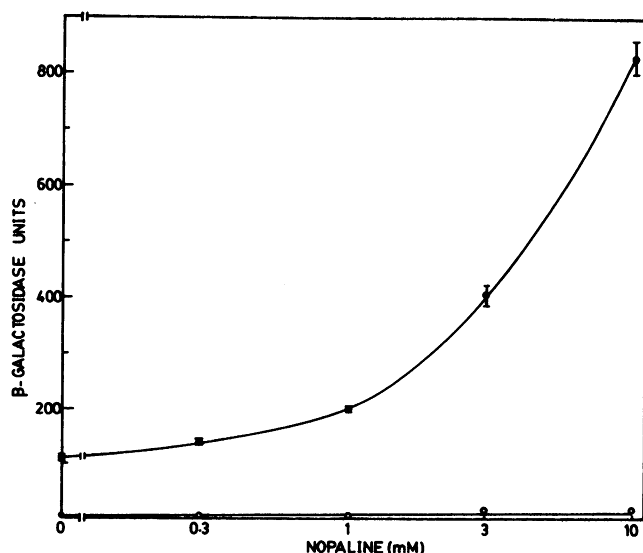

FIG. 2. Effect of nopaline concentration on the enhancement of *pinF* induction by acetosyringone. *A. tumefaciens* A348mx219 harboring a Tn*3*-HoHo1 insertion in the gene *pinF* was grown to a Klett reading of 100 in AB medium plus 0.5% glucose containing 100 μg of carbenicillin per ml, centrifuged, and suspended in supplemented K3 medium containing (●) or lacking (○) 30 μM acetosyringone and various concentrations of nopaline. After 24 h of incubation at 20°C, the cells were harvested and assayed for β-galactosidase activity.

We next investigated the effect of octopine on the acetosyringone induction of each *vir* gene in derivatives of *A. tumefaciens* A348. These strains harbored Tn*3*-HoHo1 insertions in different *vir* genes, and we thus monitored β-galactosidase activity in these strains under various incubation conditions. Each of these strains contained a *lacZ* fusion in the sole relevant *vir* gene harbored by the bacterium. The activity of *virB*, *virC*, *virD*, and *virE* in acetosyringone-induced cells was 2.5- to 5.4-fold higher in the presence of 10 mM octopine than in its absence (Table 1). As with the induction of *pinF*, octopine alone did not induce any of the *vir* genes. The β-galactosidase activity of the *virA*::*lacZ* fusion gene was not affected by either acetosyringone or octopine. The constitutive expression of this *virA*::Tn*3*-HoHo1 fusion gene in *A. tumefaciens* has been demonstrated previously by others (38). No significant induction of *virG* was seen in the presence of acetosyringone or octopine or both. The lack of induction of *virG* can be explained by the fact that the only copy of the *virG* gene present in the Ti plasmid has been disrupted in this strain. The *virG* gene product most likely functions as a transcriptional activator of itself, the other *vir* genes, and *pinF* (27, 42, 51), and this strain therefore would not contain active *virG* protein.

Neither arginine nor pyruvate, the compounds from which octopine is formed, nor the neutral amino acid leucine greatly stimulated the acetosyringone induction of *pinF* (Table 2). Arginine and leucine had some stimulatory effects on the induction of *virB*. This effect was considerably less than the effect of octopine and nopaline. We speculate that the effect of arginine and leucine on *virB* expression (and to a small extent on *pinF* expression) reflects the enrichment of the minimal K3 medium by these metabolites and the subsequent general metabolic activity of the bacterial cells. Nopaline had a greater effect on the acetosyringone induction of these genes than did octopine. This result was surprising because neither the Ti plasmid harbored by *A. tumefaciens* A348 (pTiA6) nor the chromosome in this strain (C58 chromosomal background) encodes functions that allow the catabolism of nopaline (4, 29). We therefore did not expect that nopaline should have an effect upon this bacterial strain. Figure 2 shows that, as with octopine, the enhancement of the acetosyringone induction of *pinF* by nopaline is not yet saturated at 10 mM.

Downloaded from http://jb.asm.org/ on September 21, 2018 by guest

TABLE 3. Effect of opines on induction of *vir* genes[a]

Treatment	β-Galactosidase (U)					
	virB			*pinF*		
	−AS	+AS	Fold increase over AS alone	−AS	+AS	Fold increase over AS alone
K3 medium	<0.1	27 ± 0.5		5 ± 0.4	160 ± 7.0	
K3 + octopine	<0.1	143 ± 2.4	5.3	16 ± 0.5	370 ± 5.0	2.3
K3 + nopaline	<0.1	343 ± 2.0	12.7	17 ± 0.5	829 ± 30	5.2
K3 + succinamopine	<0.1	100 ± 1.3	3.7	34 ± 2.0	450 ± 11	2.8
K3 + leucinopine	<0.1	216 ± 9.3	8.0	32 ± 2.4	487 ± 19	3.0
K3 + mannopine	<0.1	18 ± 0.6	0.7	16 ± 0.5	176 ± 7.0	1.1

[a] Acetosyringone (AS) was used at 30 μM. Opines were used at 10 mM except for nopaline, which was used at 5 mM. All values are means of four replicate experiments.

Because we observed the stimulation of acetosyringone induction of the *vir* genes and *pinF* by an opine that is not catabolized by this strain of *A. tumefaciens*, we tested the effects of other opines. Mannopine, an opine that is catabolized by *Agrobacterium* strains harboring the plasmid pTiA6 (5), did not stimulate the acetosyringone induction of *virB* or *pinF* (Table 3). Succinamopine and leucinopine at 10 mM did stimulate this induction. Neither of these opines is catabolized by *Agrobacterium* strains harboring pTiA6 (5). The "opine" acetopine, a compound that is found in certain nontransformed plant tissues (7), does not enhance the acetosyringone induction of these genes (data not shown).

We have previously shown that, following incubation of *A. tumefaciens* with tobacco protoplasts, the Ti plasmid copy number can increase relative to that of the bacterial chromosome (48). A trivial explanation for the octopine enhancement effect would be a similar increase of the Ti plasmid copy number following incubation of the bacteria with acetosyringone and octopine, thereby increasing the copy number of the *vir*::*lacZ* fusion gene in the bacterial cell. DNA blot analysis indicated, however, that such a copy number increase did not occur (data not shown).

Involvement of *virA* and *virG* in opine enhancement phenomenon. Because the transposon Tn*3*-HoHo1 acts not only as a reporter of gene activity but also as an insertional mutagen, the data presented in Table 1 suggest that the genes *virB*, *virC*, *virD*, *virE*, and *pinF* are not involved in the phenomenon of opine enhancement of *vir* gene induction. This is because the only copy of these genes has been disrupted in the relevant strains, yet octopine could still stimulate the acetosyringone induction of these genes. The participation of the genes *virA* and *virG* in the opine enhancement phenomenon was ambiguous in these experiments because the disruption of these genes would not permit acetosyringone induction.

To determine the involvement of Ti plasmid-encoded genes other than *virA* and *virG* in the potentiation of acetosyringone induction of the *vir* genes, we introduced the plasmid pSM405 into *A. tumefaciens* A136. This *Agrobacterium* strain contains the same chromosomal background as strain A348 but lacks any Ti plasmid. The plasmid pSM405 contains a Tn*3*-HoHo1 transposon in the 5′ end of the *virB* locus (42) harbored by the cosmid pVK257. This cosmid harbors the genes *pinF*, *virA*, *virB*, *virG*, *virC*, and the first two open reading frames of the *virD* locus. The acetosyringone induction of the *virB* locus in strain A136(pSM405) was enhanced approximately twofold by 10 mM octopine (Table 4). Because the first two open reading frames of the *virD* locus contained in pSM405 encode a T-DNA border-specific endonuclease (17, 41, 53), it is likely that the stimulation of the acetosyringone induction of *vir* genes requires no Ti plasmid-encoded genes other than *virA* and *virG*. These experiments do not rule out the possibility of a role for chromosomal genes in this process.

Effect of opines on T-strand synthesis and T-DNA border cleavage. We (48) and others (1, 40, 41) have shown that upon acetosyringone induction of *A. tumefaciens* cells, single-stranded T-DNA molecules, termed T-strands, accumulate in the bacteria. All possible combinations of the four T-DNA borders can be used to generate T-strands of different sizes in *A. tumefaciens* A348 (41, 48). The first two open reading frames of the *virD* region encode proteins involved in the site-specific production of nicks in the bottom strand of the T-DNA borders, leading to the production of T-strands (17, 41, 53). The proteins encoded by these two open reading frames also catalyze the double-stranded cleavage of the T-DNA at these border sequences (17). Increasing *vir* gene activity by increasing the copy number of *vir* genes resulted in increased T-strand production and double-stranded border cleavage (48). Because octopine and

TABLE 4. Effect of *vir* genes on the octopine stimulation of *vir* gene activity[a]

Strain	Gene disrupted by Tn*3*-HoHo1	Wild-type *vir* genes present in the bacterium	β-Galactosidase (U)[b]			
			K3 medium	K3 medium + acetosyringone	K3 medium + acetosyringone + octopine	Fold increase over K3 + acetosyringone
A348mx219	*pinF*	*A, B, C, G, D, E*	4 ± 0.4	84 ± 0.6	164 ± 0.6	2.0
A348mx243[c]	*virB*	*A, C, G, D, E*	4.4 ± 0.4	177 ± 0.6	355 ± 0.6	2.0
A136(pSM405)[d]	*virB*	*A, C, G*	4.4 ± 0.4	221 ± 0.6	413 ± 0.6	1.9

[a] All values are means of three replicate experiments.
[b] β-Galactosidase activity was measured after incubation at 20°C for 24 h.
[c] This strain harbors a single copy of *virB*::Tn*3*-HoHo1 that has been marker exchanged into the T1 plasmid.
[d] This strain is a merodiploid and harbors multiple copies of *virB*::Tn*3*-HoHo1.

Downloaded from http://jb.asm.org/ on September 21, 2018 by guest

nopaline enhance the acetosyringone-induced activity of *virD*, we tested whether these opines could stimulate the ability of acetosyringone to induce both the production of T-strands and the double-stranded cleavage of the T-DNA at border A.

Figure 3A shows the effect of nopaline on T-strand accumulation in *A. tumefaciens* cells. The probe, *Hin*dIII fragment 18c, hybridized with a region of the Ti-plasmid spanning the left border of T_L (Fig. 3C). This probe should thus detect T-strands between the T-DNA borders A and B (A-B), A and C (A-C), and A and D (A-D) (48). With acetosyringone at limiting concentrations (3 μM), T-strands could not be detected at these autoradiographic exposures (Fig. 3A, lanes 1 and 2) unless 5 mM nopaline was included in the incubation solution (Fig. 3A, lane 3). The use of 30 μM acetosyringone without nopaline resulted in approximately the same level of T-strand accumulation as did 3 μM acetosyringone plus 5 mM nopaline (Fig. 3A, lane 4). Inclusion of nopaline in the incubation solution resulted in increased accumulation of both the A-B and A-C T-strands (Fig. 3A, lanes 5 and 6). The photographic exposure shown in Fig. 3A was not sufficient to detect the relatively weak signal from the A-D T-strand (48).

Double-stranded cleavage of the T-DNA at border A followed by digestion of the DNA by *Eco*RI led to the production of a 3.6-kilobase-pair (kbp) fragment that could be detected when the DNA blots were hybridized with *Hin*dIII fragment 18c (Fig. 3C) (17, 47). Single-stranded DNA fragments can also be detected with this hybridization probe migrating at the equivalent mobility of 2.6 and 2.0 kbp (47, 48). Figure 3B, lanes 1 to 3, shows that such fragments could not be detected at limiting acetosyringone concentrations unless 5 mM nopaline was included in the incubation solution. At saturating acetosyringone concentrations, nopaline greatly stimulated the accumulation of these fragments in the bacterium (Fig. 3B, lanes 4 to 6). Thus, nopaline enhances the ability of acetosyringone to induce both T-strands and the double-stranded cleavage of the T-DNA borders. We obtained results similar to those described above using octopine instead of nopaline (data not shown).

Effect of nopaline and acetosyringone on plant transformation. Although we have shown that opines such as nopaline enhance the acetosyringone induction of certain *vir* genes, the production of T-strands, and the double-stranded cleavage of the T-DNA borders, we thought it important to determine whether nopaline could enhance the transformation of plants. We chose the system *G. hirsutum* in which the *A. tumefaciens*-mediated transformation efficiency is very low so that any increase in transformation efficiency by acetosyringone and opines could be easily detected. We infected *G. hirsutum* (cotton) shoot apices (46a) with *A. tumefaciens* LBA4404(pRGUS2) containing a T-DNA binary vector harboring a GUS gene. After 4 to 7 weeks in culture, tissues from two leaves originating from the primordium present at the time of shoot inoculation and two to three regenerated leaves were assayed for GUS activity by a fluorimetric assay (18).

Leaves of cotton shoot apices inoculated with *A. tumefaciens* alone or in the presence of 30 mM nopaline did not display GUS activity (Table 5). Shoot apices inoculated in the presence of 10 μM acetosyringone yielded 2 GUS-positive transformants from 57 plants tested (3.5%). Inoculation of shoot apices in the presence of nopaline plus acetosyringone, however, yielded 37 of 45 GUS-positive plants (82%). Thus, incubation of *Agrobacterium* cells in the presence of acetosyringone plus nopaline could greatly enhance the transformation of cotton.

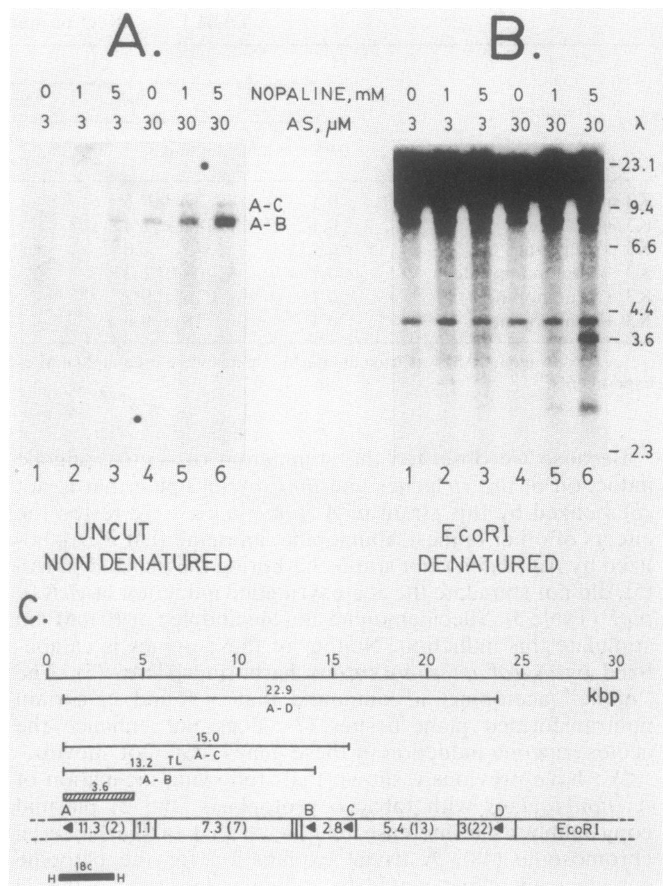

FIG. 3. Effect of nopaline on T-strand production (A) and double-stranded cleavage (B) at border A. *A. tumefaciens* A348 was grown to a Klett reading of 100 in AB medium plus 0.5% glucose, centrifuged, and suspended in K3 medium containing the indicated concentrations of acetosyringone (AS) and nopaline. After 24 h of incubation at 20°C, the cells were harvested and DNA was extracted as described previously (48). (A) Samples of 1 μg of uncut DNA were subjected to electrophoresis through a 1.0% agarose gel, transferred to nitrocellulose under nondenaturing conditions, and hybridized with ^{32}P-labeled *Hin*dIII fragment 18c (48). The A-C and A-B T-strands are indicated in the right figure margin. (B) Samples of 1.0 μg were digested with *Eco*RI, fractionated by electrophoresis through a 1.0% agarose gel, transferred to nitrocellulose under denaturing conditions, and hybridized with ^{32}P-labeled *Hin*dIII fragment 18c (48). Size markers in kilobase pairs (bacteriophage λ DNA digested with *Hin*dIII) are indicated in the right figure margin. The fragment migrating at 3.6 kbp represents the fragment generated by the double-strand cleavage at border A and at the neighboring *Eco*RI site. The fragment migrating at approximately 2.6 kbp represents a single-stranded DNA fragment released by *Eco*RI digestion (48). (C) Restriction endonuclease map of the T-DNA region of pTiA6. T-DNA border repeat sequences are indicated by ◀. The A-D, A-C, and A-B T-strands are indicated above the map. The 3.6-kbp fragment generated by the double-stranded cleavage at border A and *Eco*RI digestion is also indicated above the map. *Hin*dIII fragment 18c is indicated below the map.

Uptake of nopaline by *A. tumefaciens* A348. *A. tumefaciens* A348 harboring the C58 chromosomal background and pTiA6 does not catabolize the opines nopaline, leucinopine, or succinamopine (4, 5, 29). We were therefore surprised that these opines should have an effect on the induction of

Downloaded from http://jb.asm.org/ on September 21, 2018 by guest

TABLE 5. Transformation of cotton shoot apices by *A. tumefaciens* LBA4404(pRGUS2)

Treatment[a]	No. of shoot apices inoculated	No. of regenerated shoots assayed	No. of GUS-positive shoots[b]	% GUS-positive shoots
None	1,298	93	0	0
Nopaline	60	49	0	0
Acetosyringone	65	57	2	3.5
Nopaline + acetosyringone	60	45	37	82

[a] Nopaline was used at 30 mM. Acetosyringone was used at 10 μM. These chemicals were dissolved in dimethyl sulfoxide and applied to the shoot apex at the time of inoculation.

[b] A fluorimetric assay with 4-methyl umbelliferyl glucoronide was employed. The fluorescent methyl umbelliferone product was visualized after 24 h on a 365-nm UV light box.

the *vir* genes in this strain. Figure 4 shows, however, that strain A348 could transport nopaline when the bacteria were incubated in a medium suitable for *vir* gene induction. Preincubation of the bacteria with sodium azide abolished this uptake, indicating both that active bacterial metabolism is required for nopaline transport and that nopaline is not nonspecifically binding to the cells under these assay conditions. The lack of incorporation of ^{14}C into trichloroacetic acid-insoluble material by this strain (data not shown) indicates that [^{14}C]nopaline is not degraded to [^{14}C]arginine extracellularly, resulting in uptake by the bacterial cells of [^{14}C]arginine.

DISCUSSION

The expression of T-DNA in plant cells leads to the overproduction of the phytohormones auxin and cytokinin and to the production of novel compounds, the opines. Opines are secreted into the rhizosphere where they can be utilized by the inciting *Agrobacterium* strain as a carbon and sometimes as a nitrogen source (46). In addition, many opines can induce the ability of *Agrobacterium* species to conjugate the Ti plasmid to other, plasmidless *Agrobacterium* cells (13, 14, 20, 33). Thus, opines have heretofore been shown to serve two important functions in the infection cycle of *Agrobacterium* species: that of an energy source for the bacterium, and that as a signal molecule to stimulate the spread of the molecular disease determinant (the Ti plasmid) to *Agrobacterium* cells. In this report, we showed that certain opines can additionally serve to enhance the induction of the *vir* genes and thus may play a role in the tumorigenic process. Although the concentrations of opines used in this study to potentiate *vir* gene expression would appear to be high (1 to 10 mM), such levels can be found in crown gall tumors. Scott et al. (35) reported that the opines octopine, octopinic acid, lysopine, nopaline, and nopalinic acid could accumulate to these concentrations in various crown gall tumors. It is not known to what levels these opines accumulate in the rhizosphere. Certain Ti plasmids, however, harbor the T-DNA gene *ons*, the product of which is involved in the secretion of octopine and nopaline from crown gall tumor cells (28). Saint-Pierre and Dion (34) have determined that nopaline can accumulate to approximately 2 mM in the intercellular fluid of apple crown gall tumors.

The activity of the genes *virB*, *virG*, *virC*, *virD*, *virE*, and *pinF* was enhanced by the addition of certain opines during the period of induction (Table 1). The *Agrobacterium* strain harboring the *virA*::HoHo1 insertion used in this study expresses the *virA* gene constitutively (38). The *Agrobacterium* strain that we utilized to study *virG* activity did not contain a wild-type copy of this gene. Because the *virG* protein is most likely a transcriptional activator of itself and the other *vir* genes, it was not surprising that this strain showed no acetosyringone induction, either with or without the addition of opines. The enhancement of acetosyringone induction by opines required the genes *virA* and *virG* (Table 4); no other Ti plasmid-encoded genes were necessary. Chromosomal genes may also be involved in the enhancement phenomenon.

The extent of the enhancement of *vir* gene activity was significant—from 2- to 10-fold, depending on the *vir* gene under study and the opine used. Because the strain we used, A348, utilizes octopine and mannopine, we expected that these two opines may stimulate the acetosyringone induction of the *vir* genes. Mannopine, however, did not enhance this induction. Although octopine did stimulate the induction, the opines leucinopine, succinamopine, and nopaline were more effective. This was surprising because none of these latter opines are catabolized by this *Agrobacterium* strain. Experiments utilizing [^{14}C]nopaline indicated, however, that strain A348 harboring an octopine-type Ti plasmid can take up nopaline from the medium under the *vir* gene induction conditions utilized in this study (Fig. 4). The opines that stimulated the acetosyringone induction of the *vir* genes are all iminodiacids. Another iminodiacid, acetopine, did not enhance *vir* gene induction. This latter compound, however, can be found in the cells of some nontransformed plants (7) and is therefore not a true opine.

As well as stimulating the activity of the *vir* genes, the opines octopine and nopaline enhanced the double-stranded cleavage of the T-DNA border sequences and the production of T-strands by *Agrobacterium* species (Fig. 3). This was expected because these molecular events require the func-

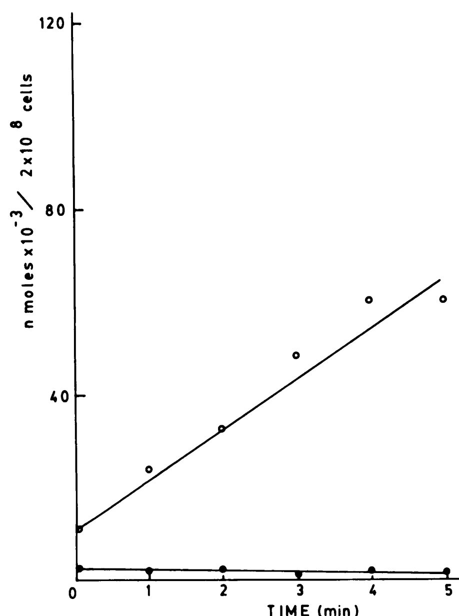

FIG. 4. Uptake of nopaline by *A. tumefaciens* A348. The bacteria were incubated in K3 medium containing [^{14}C]nopaline, either with (●) or without (○) a 1-h preincubation period in the presence of sodium azide. The bacteria were harvested and washed, and the radioactivity of the filters was counted as described in Materials and Methods.

Downloaded from http://jb.asm.org/ on September 21, 2018 by guest

tion of *virD* (1, 41, 47, 48). We previously showed that increased *vir* gene activity, caused either by induction of the bacteria by cocultivation with protoplasts rather than incubation with acetosyringone or by the addition of extra copies of *vir* genes to the bacteria, resulted in increased T-strand production (48). The data presented above suggest that the incubation of *Agrobacterium* cells with acetosyringone and opines such as nopaline results in the increased transformation efficiency of plant cells. Infection of plants with *A. tumefaciens* in the presence of acetosyringone and nopaline did significantly increase the transformation of cotton shoot tips. Infection in the presence of acetosyringone alone had only a small effect (Table 5). The treatment of *Agrobacterium* species with acetosyringone and nopaline has obvious implications with respect to the transformation of recalcitrant plant species. The promotion of the rate of crown gall tumor growth by the addition of opines has been described previously (24, 25). More recently, Whiteman Runs Him et al. (50) demonstrated the enhanced transformation efficiency of carrot root disks by *Agrobacterium rhizogenes* in the presence of octopine. Nopaline, however, did not stimulate this transformation efficiency.

The finding that *vir* gene induction is influenced by certain opines suggested to us that the genetic regulation of *vir* gene activity and those functions controlled by opines are interconnected. We are currently investigating the possibility that a number of phenomena important in the infection cycle of *Agrobacterium* species, including *vir* gene induction, opine utilization, and conjugal plasmid transfer, are under the common control of certain bacterial chromosomal genes.

ACKNOWLEDGMENTS

We thank W. Scott Chilton for providing acetopine, leucinopine, succinamopine, and [^{14}C]nopaline, Marlaya Wyncott for technical help, Laszlo Csonka, Susan Karcher, and Ann Matthysse for critical reading of the manuscript, and Marge Miles and Wilma Foust for help in preparing the manuscript.

This work was funded by a National Science Foundation Presidential Young Investigator award (DMB-835112) to S.B.G., with matching funds from Agrigenetics Research Associates. J.H.G. was supported by a grant from Cotton, Inc., to R.H.S. and by funds from the Texas Agricultural Experiment Station.

LITERATURE CITED

1. **Albright, L. M., M. F. Yanofsky, B. Leroux, D. Ma, and E. W. Nester.** 1987. Processing of the T-DNA of *Agrobacterium tumefaciens* generates border nicks and linear, single-stranded T-DNA. J. Bacteriol. **169:**1046–1055.
2. **Bevan, M. W., and M.-D. Chilton.** 1982. T-DNA of the *Agrobacterium* Ti and Ri plasmids. Annu. Rev. Genet. **16:**357–384.
3. **Bolton, G. W., E. W. Nester, and M. P. Gordon.** 1986. Plant phenolic compounds induce expression of the *Agrobacterium tumefaciens* loci needed for virulence. Science **232:**983–985.
4. **Bomhoff, G., P. M. Klapwijk, H. C. M. Kester, R. A. Schilperoort, J. P. Hernalsteens, and J. Schell.** 1976. Octopine and nopaline synthesis and breakdown genetically controlled by a plasmid of *Agrobacterium tumefaciens*. Mol. Gen. Genet. **145:** 177–181.
5. **Chilton, W. S., and M.-D. Chilton.** 1984. Mannityl opine analogs allow isolation of catabolic pathway metabolic mutants. J. Bacteriol. **158:**650–658.
6. **Christie, P. J., J. E. Ward, S. C. Winans, and E. W. Nester.** 1988. The *Agrobacterium tumefaciens virE2* gene product is a single-stranded-DNA-binding protein that associates with T-DNA. J. Bacteriol. **170:**2659–2667.
7. **Christou, P., S. G. Platt, and M. C. Ackerman.** 1986. Opine synthesis in wild-type plant tissue. Plant Physiol. **82:**218–221.
8. **Citovsky, V., G. De Vos, and P. Zambryski.** 1988. Single-stranded DNA binding protein encoded by the *virE* locus of *Agrobacterium tumefaciens*. Science **240:**501–504.
9. **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.
10. **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.
11. **Das, A.** 1988. *Agrobacterium tumefaciens virE* operon encodes a single-stranded DNA-binding protein. Proc. Natl. Acad. Sci. USA **85:**2909–2913.
12. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for Gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77:**7347–7351.
13. **Ellis, J. G., A. Kerr, A. Petit, and J. Tempé.** 1982. Conjugal transfer of nopaline and agropine Ti-plasmids—the role of agrocinopines. Mol. Gen. Genet. **186:**269–274.
14. **Ellis, J. G., P. J. Murphy, and A. Kerr.** 1982. Isolation and properties of transfer regulatory mutants of the nopaline Ti-plasmid pTiC58. Mol. Gen. Genet. **186:**275–281.
15. **Gietl, C., Z. Koukolikova-Nicola, and B. Hohn.** 1987. Mobilization of T-DNA from *Agrobacterium* to plant cells involves a protein that binds single-stranded DNA. Proc. Natl. Acad. Sci. USA **84:**9006–9010.
16. **Herrera-Estrella, L., M. De Block, P. Zambryski, M. Van Montagu, and J. Schell.** 1986. *Agrobacterium* as a vector system for the introduction of genes into plants, p. 61–93. *In* J. H. Dodds (ed.), Plant genetic engineering. Cambridge University Press, New York.
17. **Jayaswal, R. K., K. Veluthambi, S. B. Gelvin, and J. L. Slightom.** 1987. Double-stranded cleavage of T-DNA and generation of single-stranded T-DNA molecules in *Escherichia coli* by a *virD*-encoded border-specific endonuclease from *Agrobacterium tumefaciens*. J. Bacteriol. **169:**5035–5045.
18. **Jefferson, R. A.** 1987. GUS gene fusion system user's manual. Cambridge, England.
19. **Jensen, R. E., W. T. Zdybak, K. Yasuda, and W. S. Chilton.** 1977. A useful synthesis of nopaline, a crown gall tumor metabolite. Biochem. Biophys. Res. Commun. **75:**1066–1070.
20. **Kerr, A., P. Manigault, and J. Tempé.** 1977. Transfer of virulence *in vivo* and *in vitro* in *Agrobacterium*. Nature (London) **265:**560–561.
21. **Knauf, V. C., and E. W. Nester.** 1982. Wide host range cloning vectors: a cosmid clone bank of an *Agrobacterium* Ti plasmid. Plasmid **8:**45–54.
22. **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.
23. **Lichtenstein, C., and J. Draper.** 1986. Genetic engineering of plants, p. 67–119. *In* D. M. Glover (ed.), DNA cloning, vol. 2. A practical approach. IRL Press, Washington, D.C.
24. **Lippincott, J. A., and B. B. Lippincott.** 1970. Lysopine and octopine promote crown-gall tumor growth *in vivo*. Science **170:**175–176.
25. **Lippincott, J. A., B. B. Lippincott, and C.-C. Chang.** 1972. Promotion of crown-gall tumor growth by lysopine, octopine, nopaline, and carnosine. Plant Physiol. **49:**131–137.
26. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **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.
28. **Messens, E., A. Lenaerts, M. Van Montagu, and R. W. Hedges.** 1985. Genetic basis for opine secretion from crown gall tumour cells. Mol. Gen. Genet. **199:**344–348.
29. **Montoya, A. L., M.-D. Chilton, M. P. Gordon, D. Sciaky, and E. W. Nester.** 1977. Octopine and nopaline metabolism in

Downloaded from http://jb.asm.org/ on September 21, 2018 by guest

Agrobacterium tumefaciens and crown gall tumor cells: role of plasmid genes. J. Bacteriol. **129**:101–107.

30. **Nagy, J. I., and P. Maliga.** 1976. Callus induction and plant regeneration from mesophyll protoplasts of *Nicotiana sylvestris*. Z. Pflanzenphysiol. **78**:453–455.
31. **Nester, E. W., M. P. Gordon, R. M. Amasino, and M. F. Yanofsky.** 1984. Crown gall: a molecular and physiological analysis. Annu. Rev. Plant Physiol. **35**:387–413.
32. **Ooms, G., P. J. J. Hooykaas, R. J. M. Van Veen, P. Van Beelan, T. J. G. Regensburg-Tuink, and R. A. Schilperoort.** 1982. Octopine Ti-plasmid deletion mutants of *Agrobacterium tumefaciens* with emphasis on the right side of the T-region. Plasmid **7**:15–29.
33. **Petit, A., J. Tempé, A. Kerr, M. Holsters, M. Van Montagu, and J. Schell.** 1978. Substrate induction of conjugative activity of *Agrobacterium tumefaciens* Ti plasmids. Nature (London) **271**: 570–572.
34. **Saint-Pierre, B., and P. Dion.** 1988. Growth of nopaline-utilizing *Agrobacterium* and *Pseudomonas* in extracts of crown-gall tumors. Can. J. Microbiol. **34**:793–801.
35. **Scott, I. M., J. L. Firmin, D. N. Butcher, L. M. Searle, A. K. Sogeke, J. Eagles, J. F. March, R. Self, and G. R. Fenwick.** 1979. Analysis of a range of crown gall and normal plant tissues for Ti plasmid-determined compounds. Mol. Gen. Genet. **176**:57–65.
36. **Stachel, S. E., G. An, C. Flores, and E. W. Nester.** 1985. A Tn*3* *lacZ* transposon for the random generation of β-galactosidase gene fusions: application to the analysis of gene expression in *Agrobacterium*. EMBO J. **4**:891–898.
37. **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.
38. **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.
39. **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.
40. **Stachel, S. E., B. Timmerman, and P. Zambryski.** 1986. Generation of single-stranded T-DNA molecules during the initial stages of T-DNA transfer from *Agrobacterium tumefaciens* to plant cells. Nature (London) **322**:706–712.
41. **Stachel, S. E., B. Timmerman, and P. Zambryski.** 1987. Activation of *Agrobacterium tumefaciens vir* gene expression generates multiple single-stranded T-strand molecules from the pTiA6 T-region: requirement for 5′ *virD* gene products. EMBO J. **6**:857–863.
42. **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.
43. **Stachel, S. E., and P. C. Zambryski.** 1986. *Agrobacterium tumefaciens* and the susceptible plant cell: a novel adaptation of extracellular recognition and DNA conjugation. Cell **47**:155–157.
44. **Tait, R. C., and C. I. Kado.** 1988. Regulation of the *virC* and *virD* promoters of pTiC58 by the *ros* chromosomal mutation of *Agrobacterium tumefaciens*. Mol. Microbiol. **2**:385–392.
45. **Tempé, J., and A. Goldmann.** 1982. Occurrence and biosynthesis of opines, p. 428–449. *In* G. Kahl and J. S. Schell (ed.), Molecular biology of plant tumors. Academic Press, Inc., Orlando, Fla.
46. **Tempé, J., and A. Petit.** 1982. Opine utilization by *Agrobacterium*, p. 451–459. *In* G. Kahl and J. S. Schell (ed.), Molecular biology of plant tumors. Academic Press, Inc., Orlando, Fla.

46a. **Ulian, E. C., R. H. Smith, J. H. Gould, and T. D. McKnight.** 1988. Transformation of plants via the shoot apex. In Vitro Cell. Dev. Biol. **24**:951–954.

47. **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.
48. **Veluthambi, K., W. Ream, and S. B. Gelvin.** 1988. Virulence genes, borders, and overdrive generate single-stranded T-DNA molecules from the A6 Ti plasmid of *Agrobacterium tumefaciens*. J. Bacteriol. **170**:1523–1532.
49. **Wang, K., S. E. Stachel, B. Timmerman, M. Van Montagu, and P. C. Zambryski.** 1987. Site-specific nick in the T-DNA border sequence as a result of *Agrobacterium vir* gene expression. Science **235**:587–591.
50. **Whiteman Runs Him, K., R. J. Scheffer, and G. A. Strobel.** 1988. Factors influencing root formation in dicots by *Agrobacterium rhizogenes*. Can. J. Bot. **66**:642–644.
51. **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.
52. **Yamamoto, A., M. Iwahashi, M. F. Yanofsky, E. W. Nester, I. Takebe, and Y. Machida.** 1987. The promoter proximal region in the *virD* locus of *Agrobacterium tumefaciens* is necessary for the plant-inducible circularization of T-DNA. Mol. Gen. Genet. **206**:174–177.
53. **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.

Downloaded from http://jb.asm.org/ on September 21, 2018 by guest