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Expression of an *Agrobacterium* Ti Plasmid Gene Involved in Cytokinin Biosynthesis Is Regulated by Virulence Loci and Induced by Plant Phenolic Compounds

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The nopaline-type Ti plasmid T37 of *Agrobacterium tumefaciens* carries two distinct genes that encode enzymes involved in cytokinin biosynthesis. In this report, we show that the level of expression of one of these genes was increased dramatically by culture conditions that increased the expression of Ti plasmid virulence genes, including coculture with plant cells or treatment with acetosyringone, a plant phenolic compound. When this nopaline-type Ti plasmid gene was introduced into *Agrobacterium* strains containing an octopine-type Ti plasmid, similar induction of expression by culture conditions was observed, and analysis of virulence region mutants demonstrated that this induction was under the control of the *virA* and *virG* regulatory loci. We further show that induction was strongly pH dependent in octopine strains but, under the conditions examined, pH independent in nopaline strains.

Agrobacterium tumefaciens causes crown gall tumor formation on many species of plants. The ability of *A. tumefaciens* to transform plant cells neoplastically is conferred by the Ti (tumor-inducing) plasmid (12, 24).

Two regions of the Ti plasmid participate in tumor formation, the T-DNA (transferred DNA) and virulence (*vir*) regions. Plant cells are converted to a tumorous state when the T-DNA segment of the Ti plasmid is transferred to the plant cell and integrated into the nuclear genome, and certain T-DNA oncogenes are transcribed (13, 24). These oncogenes encode enzymes that direct the biosynthesis of the plant growth factors indole-3-acetic acid (an auxin) and zeatin (a cytokinin) (1, 7, 9, 26, 33). The *vir* region is operationally defined as the region of the Ti plasmid necessary for T-DNA transfer to the plant cell and perhaps for integration and expression of T-DNA (i.e., for tumor formation) (16, 18, 19, 29). Most *vir* genes are not expressed unless agrobacteria come in contact with exudates of plant cells (30). The *vir*-inducing activity of plant exudates can be mimicked by acetosyringone and other plant phenolic compounds (28). The induction of *vir* genes on the octopine-type Ti plasmid is mediated through two *vir* loci, *virA* and *virG*, which are thought to act in concert, respectively, as an environmental sensor of contact with plant cells and a transducer that converts this environmental cue into transcriptional activation of other *vir* genes (32, 37).

The T-DNA gene directing the production of cytokinins in crown gall tumor cells encodes a transferase that attaches the Δ^2-isopentenyl group of Δ^2-isopentenyl pyrophosphate (dimethylallylpyrophosphate) to the N-6 position of AMP (1, 7, 9). This enzymatic activity was first described in extracts of normal plant tissue and has been designated, in abbreviated form, isopentenyl transferase (Ipt) (10). Recently, it has been demonstrated that within the *vir* regions of the nopaline-type Ti plasmids C58 and T37 there is a locus, designated *tzs*, that encodes an Ipt activity (2, 8). Thus, these Ti plasmids contain two genes that encode similar enzymes; however, the nucleotide and predicted amino acid sequences of these genes are surprisingly divergent for two linked genes which encode proteins with similar functions (2, 8). The role of the *vir* region *ipt* locus is not known. In this report, we show that expression of this *ipt* gene is induced in a manner similar to that of other *vir* loci.

MATERIALS AND METHODS

Plasmid constructions. Standard procedures were used for plasmid DNA preparation, transformation, and screening (20). Plasmid pAL116 was constructed by isolating the pTiT37 *tzs* locus as a 1.4-kilobase *Bam*HI-*Hin*dIII fragment from *Bam*HI- and *Hin*dIII-digested pDA1-12 (3). This fragment contains a 1.4-kilobase *Hpa*I-*Hin*dII fragment of pTiT37 DNA and an additional 9 nucleotides from the pUC18 polylinker between the *Hin*cII and *Bam*HI sites. This fragment was ligated into the binary Ti plasmid vector pGA583 (6) which was previously digested with *Hin*dIII and *Bgl*II. The plasmid was transferred to *A. tumefaciens* strains by triparental mating as was previously described (6). A map of this plasmid is presented in Fig. 1.

An SP65 *tzs* construct was generously provided by D. E. Akiyoshi and consists of nucleotides 152 to 988 of the pTiT37 *tzs* locus (see reference 2 for nucleotide sequence and numbering) inserted into the *Eco*RI site of the vector SP65 (Promega Biotec, Madison, Wis.).

Bacterial and plant cell cultures. *A. tumefaciens* A208 and A348 consist of the A136 chromosomal background containing, respectively, the T37 and A6 Ti plasmids (18, 22); strains A348A$^-$, A348E$^-$, and A348G$^-$ are, respectively, *vir* mutants 226, 358, and 19 which have been described by Stachel and Nester (29); *A. rhizogenes* A4 has been described previously (36). *Agrobacterium* strains were maintained on AB minimal agar (13) with antibiotic selection done where appropriate. The origin and weekly subculture regimen of the *Nicotiana tabacum* suspension culture used in this study have been described before (5).

For *tzs* induction, *Agrobacterium* strains were inoculated into 1 ml of the liquid medium to be used for induction (see below) and cultured with agitation at 28°C to an optical density at 600 nm (OD_{600}) of 1 to 1.5. The cells were

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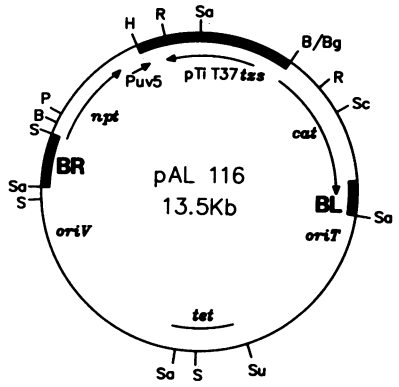

FIG. 1. Physical map of pAL116. The *tzs* locus of the T37 Ti plasmid was subcloned into binary Ti plasmid vector pGA583 as described in Materials and Methods. Symbols: pTiT37 *tzs*, restriction fragment of the pTiT37 Ti plasmid containing the *tzs* locus as described in Materials and Methods; → and ←, directions of transcription. B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; P, *Pst*I; R, *Eco*RI; S, *Sst*II; Sa, *Sal*I; Sc, *Sca*I; Su, *Stu*I; *Puv5*, *lacUV5* promoter; *oriT*, RK2 origin of transfer; *oriV*, RK2 origin of replication; *npt*, neomycin phosphotransferase gene of Tn*5*; *cat*, chloramphenicol acetyl transferase gene, BR and BL, right and left T-DNA borders.

inoculated to an OD_{600} of 0.1 into various media with or without 100 μM acetosyringone from a stock of 0.5 M acetosyringone in 50% (vol/vol) dimethyl sulfoxide. Control cells received an equal portion of 50% dimethyl sulfoxide. The cells were cultured for 6 h, and then another 100 μM portion of acetosyringone or 50% dimethyl sulfoxide was added. After 3 additional hours of culture, the cells were harvested for RNA extraction as described below. For *tzs* induction by coculture with plant cells, *Agrobacterium* strains were inoculated to an OD_{600} of 0.1 into an *N. tabacum* cell suspension culture. The *N. tabacum* culture was used 3 days after subculture, as described previously (5). After 10 h of coculture, the plant cells were removed by centrifugation at 30 × *g* for 5 min. The bacteria were pelleted from the supernatant by centrifugation at 2,000 × *g* for 5 min and suspended in 4 ml of AB medium. RNA was extracted immediately thereafter. All induction experiments were repeated on independent cultures two or more times.

Mannitol-glutamate medium (MG/L) and AB minimal medium were prepared as described previously (13, 17), and the pH was adjusted with HCl or NaOH. AB medium was supplemented with 2 g/liter of Casamino Acids (Difco Laboratories, Detroit, Mich.) where indicated.

RNA isolation. To 4 ml of an actively growing suspension of agrobacteria in a culture flask (OD_{600} of 1.0 to 1.4), 3 ml of an emulsion consisting of phenol (preequilibrated with 1 M Tris hydrochloride to pH 7) and extraction buffer (1 M NaCl, 0.2 M Tris hydrochloride [pH 7.5], 0.05 M EDTA, 5% sodium dodecyl sulfate, 0.5% β-mercaptoethanol) (2:1 [vol/vol]) was added. The flask was rapidly transferred to a shaking water bath at 60°C and agitated for 3 to 5 min. The emulsion was then transferred to a centrifuge tube containing 2 ml of chloroform-isobutanol (24:1), vortexed, and centrifuged at 17,000 × *g* for 15 min. The upper aqueous phase was removed and precipitated with 2.5 volumes of ethanol. The nucleic acid pellet was suspended in CE (10 mM sodium citrate–1 mM EDTA [pH 7.0] treated with 0.05% diethyl pyrocarbonate), brought to 2.5 M ammonium acetate, and precipitated with 2.5 volumes of ethanol. The precipitate was then suspended in CE and quantitated by UV absorbance.

RNA electrophoresis and blot hybridization. A total of 1 μg of nucleic acid (the isolation procedure resulted in some DNA in the RNA sample) was brought to 0.2% sodium dodecyl sulfate–2% Ficoll–2% glycerol–1 mM EDTA–0.02% bromophenol blue in CE, heated to 65°C for 5 min, and electrophoresed at 7 V/cm through a 1.2% agarose Tris-borate-EDTA gel (19). The gel was stained in 0.5 μg of ethidium bromide per ml, photographed, and blotted overnight with CE as the transfer buffer onto a nylon membrane. The membrane was baked at 65°C for 30 min and hydridized as previously described (4) with the following modifications. The hybridization solution consisted of 0.25 M $NaHPO_4$, 5% sodium dodecyl sulfate, 0.5% nonfat dry milk, 2.5 mM EDTA, 10% (wt/vol) polyethylene glycol (molecular weight, 8,000), 25 μM aurintricarboxylic acid, and 25 μg of carrier yeast RNA per ml. The blot was prehybridized with this solution for 4 h at 65°C, and then 5 × 10^5 cpm of SP6 *tzs* probe per ml was added. Hybridization was allowed to continue for 15 h at 65°C. The blots were then washed extensively in 0.25 M $NaHPO_4$–1% sodium dodecyl sulfate–1 mM EDTA at 65°C and exposed to Kodak XAR film (Eastman Kodak Co.) for 15 h at room temperature.

RNA probe synthesis. Cleavage of SP65 *tzs* by *Bam*HI and transcription by SP6 polymerase (Promega Biotec) results in an antisense transcript complementary to 112 bases of 5′ noncoding and all of the coding regions (except for the last six bases at the 3′ end) of the pTiT37 *tzs* gene. The probe reaction consisted of 250 ng of template DNA; 1× transcription buffer (Bethesda Research Laboratories, Inc., Gaithersburg, Md.); ATP, CTP, and GTP (500 μM each); 25 μCi [α-^{32}P]UTP (400 Ci/mmol, 10 mCi/ml; Amersham Corp., Arlington Heights, Ill.); 7.5 U of RNAguard (Pharmacia); 5 mM dithiothreitol; and 4 U of SP6 RNA polymerase (Bethesda Research Laboratories) in a volume of 6 μl and was carried out at 40°C for 15 min. The reaction was simultaneously stopped and the probe was precipitated by the addition of 5 μg of tRNA, 100 μl of 2.5 M ammonium acetate, and 250 μl of ethanol. The probe was then pelleted and suspended in CE.

Enzymes and reagents. Reagents were obtained from New England BioLabs, Inc., Beverly, Mass.; Sigma Chemical Co., St. Louis, Mo.; and J. T. Baker Chemical Co., Philadelphia, Pa., in addition to the suppliers listed in Materials and Methods. Components of bacterial media were from Difco. Acetosyringone (3′,5′-dimethoxy-4′-hydroxyacetophenone) was from Aldrich Chemical Co., Inc., Milwaukee, Wis.

RESULTS

Induction of pTiT37 *tzs* expression. The A208 strain of *A. tumefaciens* containing the wild-type T37 Ti plasmid was grown in MG/L medium with or without acetosyringone, and RNA prepared from these cultures was fractionated by nondenaturing agarose gel electrophoresis and capillary blotted onto nylon membranes. The membranes were hybridized to a ^{32}P-labeled antisense RNA probe complementary to the *tzs* gene. The presence of acetosyringone in the culture medium resulted in the accumulation of high levels of mRNA homologous to the *tzs* gene (Fig. 2, lane 2). Under identical conditions of hybridization and autoradiographic exposure, no mRNA corresponding to the *tzs* gene was detectable in RNA prepared from cultures grown without acetosyringone (Fig. 2, lane 1). Since a 10-fold reduction in the quantity of RNA per lane from induced cultures gave a strong positive autoradiographic signal (data not shown), we estimate that

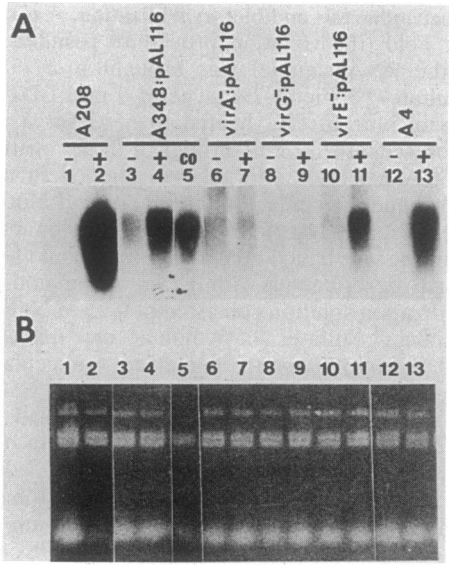

FIG. 2. Analysis of steady-state levels of *tzs* mRNA. Total RNA was prepared from *A. tumefaciens* and electrophoresed as described in Materials and Methods. (A) Autoradiogram of a Northern (RNA) blot of total RNA hybridized to a uniformly ^{32}P-labeled RNA probe complementary to the *tzs* mRNA. Symbols: +, lanes contain RNA from cultures grown with acetosyringone; −, lanes from cultures grown without acetosyringone; CO, lane contains RNA from bacteria cocultured with plant cells. (B) Ethidium-stained agarose gel lanes corresponding to the lanes of the autoradiogram demonstrate approximately equal amounts of RNA loaded in each lane. Lanes contain RNA from the following bacterial cultures. Lanes: 1, A208 cultured in MG/L (pH 7.0) without acetosyringone; 2, as in lane 1 but with acetosyringone; 3, A348 harboring pAL116 cultured in AB with Casamino Acids at pH 5.6 without acetosyringone; 4, as in lane 3 with acetosyringone; 5, as in lane 3 cocultured with plant cells; 6, *virA* mutant harboring pAL116 cultured as in lane 3; 7, as in lane 6 with acetosyringone; 8, *virG* mutant harboring pAL116 cultured as in lane 3; 9, as in lane 8 with acetosyringone; 10, *virE* mutant harboring pAL116 cultured as in lane 3; 11, as in lane 10 with acetosyringone; 12, *A. rhizogenes* A4 cultured as in lane 3; 13, as in lane 12 with acetosyringone.

the steady-state levels of *tzs* mRNA were increased at least 50-fold by growth of the bacteria in the presence of acetosyringone.

***virA*- and *virG*-regulated induction of the pTiT37 *tzs* locus in the presence of an octopine-type Ti plasmid.** To test whether expression of a locus from a nopaline-type Ti plasmid is regulated properly in the presence of an octopine-type Ti plasmid, a segment of pTiT37 DNA consisting of the *tzs* coding sequence bounded by 263 nucleotides 5′ of the start of translation and 400 base pairs 3′ of the stop codon was cloned into the binary vector pGA583 (6), resulting in plasmid pAL116 (Fig. 1). In this construct, the *tzs* locus is within the T-DNA borders and is oriented such that *tzs* transcription is directed towards the *lacUV5* promoter. Plasmid pAL116 was introduced into *A. tumefaciens* A348 (harboring the A6 octopine-type Ti plasmid), and cultures of this strain were grown in AB medium with Casamino Acids (pH 5.6) in the presence or absence of acetosyringone. Analysis of RNA from these cultures demonstrated acetosyringone-inducible expression of the T37 *tzs* locus (Fig. 2, lanes 3 and 4). The lack of induction of *tzs* gene expression when Ti plasmid-less strain A136 harboring pAL116 was cultured with acetosyringone (data not shown) demonstrated that expression of this locus is dependent on other Ti plasmid-borne genes.

To determine whether the regulation of pTiT37 *tzs* expression was under the control of the regulatory loci *virA* and *virG*, which control expression of other octopine-type Ti plasmid *vir* genes (32), pAL116 was introduced into insertion mutants of *A. tumefaciens* A348 containing lesions in the *virA*, *virE*, and *virG* loci (29), and induction was measured. In the *virE* mutant background, normal induction of the *tzs* locus by acetosyringone was observed (Fig. 2, lanes 10 and 11). However, in the *virA* and *virG* mutant backgrounds, *tzs* induction did not occur (Fig. 2, lanes 6 through 9). Thus, induction of this nopaline-type Ti plasmid locus by plant phenolic compounds is under the control of the *virA* and *virG* regulatory genes of the octopine-type Ti plasmid in these cells.

To determine whether induction of the *tzs* gene by pure acetosyringone is similar to the level of induction that would occur in the presence of plant cells, strain A348 containing pAL116 was cocultured with an actively growing tobacco suspension culture (5) for 10 h in Murashige-Skoog (23) medium. The level of *tzs* induction by coculture was similar to that observed with acetosyringone in the bacterial medium (Fig. 2, lane 5).

In the pAL116 construct, the *tzs* gene is within the region of the vector transferred to plant cells. Thus, it was possible that the positive signal on nondenaturing RNA blots was due to formation of a single-stranded transfer intermediate of T-DNA (31). However, this possibility is unlikely, since the size range of the hybridizing material was identical, regardless of whether the *tzs* gene was in the T-DNA of pAL116 or in the *vir* region of pTiT37, and comparison to molecular weight markers indicates that the size of this monocistronic transcript was 1,050 nucleotides, approximately the size of the *tzs* coding region (2). Furthermore, treatment of the samples with RNAse before electrophoresis eliminated the induced hybridizing material (data not shown).

More stringent growth conditions necessary for *tzs* induction for octopine-type than for nopaline-type Ti plasmids. For our initial observations of *tzs* inducibility in strain A208, RNA was isolated from cultures grown in rich (MG/L) medium at pH 7.0. However, numerous attempts at obtaining induction under similar culture conditions with the *tzs* locus present on pAL116 and in strain A348 were unsuccessful. Stachel et al. (30) have reported that octopine-plasmid *vir* gene induction is pH dependent, with an optimum at pH 5.2 to 5.4. Lowering the pH of rich MG/L medium from 7.0 to 5.6 did not result in *tzs* induction in A348, but induction in A208 was similar to that obtained at pH 7.0. However, after three to four doublings of a culture of either strain A208 or A348 to an OD_{600} of approximately 1, this medium was conditioned to a pH above neutral. A combination of lowered pH and a minimal medium with higher buffering capacity (AB medium with or without Casamino Acids) was thus tried; this medium resulted in maintenance of the initial pH throughout the culture period and *tzs* induction in the octopine-type strain (Table 1). We believe that the lower RNA levels observed with AB minimal medium compared with AB medium with amino acids (Table 1) result from a reduction in the ratio of mRNAs to more abundant and stable RNAs (e.g., rRNAs) when the cells grow slowly in minimal medium and does not reflect attenuated induction. These results, summarized in Table 1, indicate that the conditions optimal for induction and accumulation of *tzs* mRNA, and perhaps other *vir* genes, are less pH dependent when the regulation is under nopaline-type Ti plasmid control, rather than octopine-type Ti plas-

TABLE 1. Relative levels of *tzs* mRNA[a] induction by acetosyringone in *A. tumefaciens* A208(pTiT37) and A348(pTiA6) containing pAL116[b]

Strain	*tzs* induction level in medium				
	MG/L (pH 7.0)	MG/L (pH 5.6[c])	AB (pH 7.0)	AB (pH 5.6)	AB/CAA[d] (pH 5.6)
A208	+++	+++	++	++	+++
A348(pAL116)	ND[e]	ND	ND	+	++

[a] mRNA levels were derived from the intensity of the autoradiographic signal from a Northern blot exposed in the linear range of the XAR film.
[b] Various media were used to grow the strains; the composition of each is given in Materials and Methods.
[c] The pH was greater than 7.0 after growth of *A. tumefaciens* in this medium.
[d] CAA, Casamino Acids.
[e] ND, Not detected during a 15-h exposure.

mid control. Whether this difference between *tzs* regulation by octopine- and nopaline-type plasmids is the result of differences in the *virA* and *virG* loci is unknown but readily testable.

We also observed lower steady-state levels of the *tzs* mRNA in A348 than in A208 (Fig. 2 and Table 1). This observation may be due to the orientation of the *tzs* locus in pAL116 (i.e., facing the *lacUV5* promoter within the T-DNA region that is undergoing mobilization) or due to the lack of a complete *tzs* promoter in this construct. Alternatively, the inductive machinery of octopine- and nopaline-type Ti plasmids may have diverged sufficiently so that the system encoded by the octopine-type Ti plasmid failed to fully activate this nopaline-type Ti plasmid gene.

Induction of *tzs*-homologous sequences in *A. rhizogenes*. It has recently been reported (3) that strains of *A. rhizogenes* containing the A4 Ri plasmid produce zeatin and contain DNA sequences which are homologous to the pTiT37 *tzs* locus. Therefore, we examined induction of the A4 Ri plasmid *tzs* locus by acetosyringone. Strain A4 of *A. rhizogenes* was cultured with or without acetosyringone in AB minimal medium supplemented with Casamino Acids (pH 5.6), and blot analysis of RNA extracted from these cultures was performed as described above. As shown in Fig. 2 (lanes 12 and 13), the expression of a sequence of the Ri plasmid homologous to the *tzs* locus was induced by acetosyringone.

DISCUSSION

The results presented here show that expression of the *tzs* locus on the *A. tumefaciens* nopaline-type T37 Ti plasmid and a related locus on the *A. rhizogenes* A4 Ri plasmid was induced by the phenolic compound acetosyringone (Fig. 2). This compound and related phenolic compounds have been shown to induce the expression of the virulence genes and another locus of unknown function (*pinF*) on an octopine-type Ti plasmid (29). When a DNA fragment containing the pTiT37 *tzs* gene was placed in a binary vector and introduced into *A. tumefaciens*, this locus retained inducibility by phenolic compounds and coculture with plant cells in the presence, but not in the absence, of the A6 octopine-type Ti plasmid (Fig. 2). This result demonstrates that 1.4 kilobases of DNA containing this locus was sufficient to confer induction. However, the induced levels of *tzs* RNA transcribed from a binary vector in this octopine strain were two- to threefold lower than the levels detected in a nopaline strain. This finding could be the result of a requirement for additional flanking sequences or because of differences between the octopine- and nopaline-type Ti plasmid *virA* and *virG* loci, but a more likely explanation is that it is because of the configuration of the subclone tested (see Results).

Induction of the pTiT37 *tzs* gene in *Agrobacterium* strains containing an octopine-type Ti plasmid is mediated by the *virA* and *virG* loci in a manner analogous to other octopine-type Ti plasmid *vir* loci (Fig. 2, lanes 3 to 11). There are, however, differences in the pH requirements for *tzs* induction between octopine- and nopaline-type Ti plasmids. Induction occurs in octopine strains only in medium at a low pH, whereas induction in nopaline strains occurs in medium at low and neutral pHs (Table 1). Since the chromosomal backgrounds of these strains are identical, these differences must be Ti plasmid encoded. It would be interesting to complement octopine-nopaline-type Ti plasmid mutants with nopaline-octopine-type Ti plasmid loci and to examine induction differences to determine whether these differences reside in specific *vir* loci.

Recently, it has been reported that *Agrobacterium* cultures, preinduced with acetosyringone, transform plant cells at higher frequencies than uninduced cultures do (27). For this study, a nopaline-type *Agrobacterium* strain grown in rich, unbuffered medium at an initial pH of 5.6 was used. We have observed that the pH of a similar medium will rise during the course of culture growth; however, the use of a nopaline strain may eliminate the low pH requirement for *vir* induction and enhanced transformation. The use of preinduction with *Agrobacterium* strains containing octopine-type Ti plasmids to increase transformation efficiency may benefit from the use of a low-pH medium with a high buffering capacity (e.g., AB medium with Casamino Acids) or from supplementing other rich media with sufficient buffering capacity to maintain a pH below 5.6 for the duration of the culture period.

The function(s) of the *tzs* locus in tumor formation is unknown. That this locus has evolved to respond to induction by plant cells and is conserved in certain *A. tumefaciens* and *A. rhizogenes* strains suggests that the product of the enzyme encoded by this gene, a cytokinin, plays a role in the early stages of tumorigenesis. Cytokinins stimulate cell division, and plant cells that are rapidly dividing are maximally competent for *Agrobacterium*-mediated transformation (5). Furthermore, there are specific stages of the plant cell cycle at which the frequency of integration and expression of foreign DNA is highest (21). Preliminary evidence from our lab indicates that dividing cells express T-DNA oncogenes at higher levels than nondividing cells do (unpublished results). Thus, possible roles, which are not exclusive, for plant-inducible cytokinin production by *A. tumefaciens* are (i) to condition plant cells to a state in which the transfer of DNA from the bacteria to the plant cell is optimal, (ii) to ensure that plant cells pass through stages of the cell cycle in which DNA integration can occur, or (iii) to stimulate high levels of postintegration T-DNA expression, thus leading to rapid tumor development. As suggested previously (2, 8), cytokinin production may also affect the host range of *Agrobacterium* spp.

It has been reported that production of the cytokinin zeatin occurs in strains of *A. tumefaciens* containing nopa-

line- but not octopine-type Ti plasmids (8, 25). It has also been suggested that zeatin production by certain *Agrobacterium* strains is probably not essential for virulence, since octopine- and nopaline-type Ti plasmids both confer high levels of virulence (8). In agreement with this view, our own attempts to detect DNA sequences homologous to the coding region of the pTiT37 *tzs* locus in octopine-type Ti plasmids have been unsuccessful (unpublished results). However, it has been reported that with certain culture conditions, the B6 octopine-type Ti plasmid is responsible for zeatin production in *A. tumefaciens* spp. (14, 35), and the presence of an octopine-type Ti plasmid confers high levels of isopentenyladenine production to *Rhizobium leguminosarum* (34). Transcription of certain T-DNA genes does occur in *A. tumefaciens* (15), and it is possible that octopine-type, Ti plasmid-mediated cytokinin production in *A. tumefaciens* results from transcription of the T-DNA *ipt* locus. However, since the cultural requirements for *tzs* induction are more stringent for octopine than for nopaline *Agrobacterium* strains, it is also possible that octopine-type Ti plasmids do contain a locus outside of the T-DNA that directs cytokinin production, and the conflicting data on cytokinin production by octopine strains may be due to the different culture conditions used by different groups. Our current knowledge of the conditions required for *tzs* expression will allow an examination of these possibilities.

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ADDENDUM IN PROOF

T. Hirooka et al. (J. Bacteriol. **169**:1529–1536) and P. M. Rogowsky et al. (J. Bacteriol. **169**:5101–5112) have reported that the expression of certain virulence genes of the nopaline-type pTiC58 plasmid is regulated by *virA* and *virG* and induced by acetosyringone.

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