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Inducible Expression of Cytokinin Biosynthesis in *Agrobacterium tumefaciens* by Plant Phenolics

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Nopaline strains of *Agrobacterium tumefaciens* contain a gene, *tzs*, that encodes a cytokinin biosynthetic prenyl transferase. The gene is located adjacent to the Ti plasmid virulence region and is constitutively expressed at low levels. As a result, bacteria containing *tzs* secrete low levels of zeatin into the medium. We find zeatin secretion to be induced more than 100-fold by acetosyringone, one of a number of naturally occurring phenolics produced by plants in response to wounding. Induction was very sensitive to the pH of the medium (optimum pH 5.5) and was due to massive overexpression of *tzs*-encoded cytokinin prenyl transferase activity. The relative ability of members of a set of phenols to induce *tzs* expression was examined and found to be parallel to that reported for activation of other virulence genes. A series of molecular cloning experiments established that *virA* and *virG*, two genes known to be essential to the virulence induction process, were necessary and sufficient for phenolic-induced *tzs* expression. Sequences present in the promoter region of *tzs* were found to be similar to those present in genes regulated by bacterial two-component positive regulatory systems.

Additional keywords: crown gall, dimethylallyl transferase, *vir* gene regulation.

Agrobacterium tumefaciens is a soil bacterium that, on many dicotyledonous plants, causes the growth of tumors called crown galls. Onset of the disease occurs in wounded plant tissues by transfer of a specific segment (the T-DNA) from *A. tumefaciens*' large tumor-inducing (Ti) plasmid (Fig. 1A) to the plant cell (Chilton *et al.* 1977; Nester and Kosuge 1981; Panopoulos and Peet 1985). Located on the T-DNA are genes that encode auxin and cytokinin biosynthetic enzymes. When incorporated into the plant genome and expressed by plant cells, these genes are responsible for the appearance of the tumorous morphology (Nester *et al.* 1984; Morris 1986; Thomashow *et al.* 1986). Transfer of the T-DNA is mediated by an adjacent segment of the Ti plasmid called the virulence (*vir*) region, which is composed of several genetic loci that are expressed during the early stages of infection (Hille *et al.* 1982; Klee *et al.* 1983, Hille *et al.* 1984; Hooykaas *et al.* 1984; Stachel and Nester 1986).

Expression of the *vir* region genes is a tightly regulated process. During normal bacterial growth this expression is very low, but when *Agrobacterium* cells are cultivated in the presence of plant cell exudates, expression of several *vir* loci is induced to substantially higher levels (Stachel *et al.* 1985; Stachel and Zambryski 1986a). This induction was shown to be caused by a number of low molecular weight plant phenolics, the most active of which is acetosyringone (AS), a compound secreted naturally from wounded tobacco tissues and also present in tobacco cell cultures (Stachel *et al.* 1985; Bolton *et al.* 1986). Two virulence genes, *virA* and *virG*, were shown to control the expression of several virulence loci, including *virB*, *C*, *D*, and *E* (Winans *et al.* 1986; Veluthambi *et al.* 1987; Leroux *et al.* 1987), in what appears to be a classical prokaryotic two-component regulatory system.

Studies of other two-component regulatory systems, controlling processes as diverse as osmotic regulation (*E. coli ompR/envZ* system; Hall and Silhavy 1981), phosphate limitation (*E. coli phoB/phoR* system; Guan *et al.* 1983; Shinagawa *et al.* 1983), and nitrogen assimilation and metabolism (*K. pneumoniae ntrB/ntrC* system; McFarland *et al.* 1981), have led to the development of a model illustrating phenolic-induced expression of *vir* genes. Nucleotide sequence analyses of the *virA* and *virG* genes from *A. tumefaciens* revealed substantial similarity to genes present in all of the two-component regulatory systems listed above and, by analogy, to these other systems, provide insight into how the expression of virulence functions might be induced by plant phenolics (Winans *et al.* 1986; Leroux *et al.* 1987; Melchers *et al.* 1986; Ronson *et al.* 1987; Powell *et al.* 1987). By examination of the deduced amino acid sequence of virA (Leroux *et al.* 1987), and comparison with the gene products from the other regulatory systems, it now appears that the *virA* gene product is probably an inner membrane protein that recognizes specific signal molecules like acetosyringone. This, in some as yet undetermined manner, activates *virG*, causing transcription of the inducible virulence loci to increase by as much as several 100-fold.

A. tumefaciens strains contain genes that encode enzymes involved in cytokinin biosynthesis (Barry *et al.* 1984; Beaty *et al.* 1986). The first gene that was identified, *tmr*, is present in all octopine and nopaline strains examined thus far and encodes dimethylallyl pyrophosphate:5'AMP dimethylallyl transferase (DMA transferase), a cytokinin prenyl transferase that catalyzes the synthesis of isopentenyl-adenosine 5'-phosphate (iPMP) from dimethylallyl pyrophosphate (DMAPP) and 5'-AMP (Akiyoshi *et al.* 1984; Barry *et al.* 1984). This reaction is illustrated in Figure 1B. The *tmr* gene contains a eukaryotic-type promoter and is expressed only in transformed plant tissues (Barry *et al.* 1984; Buchmann *et al.* 1985). A second gene, *tzs*, is present

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only on nopaline Ti plasmids where it is located adjacent to the virulence region (Regier and Morris 1982). This gene, like *tmr*, also encodes DMA transferase activity (Akiyoshi *et al.* 1985; Beaty *et al.* 1986; Akiyoshi *et al.* 1987). Although the *tzs* open-reading frame shares 60% nucleotide identity with *tmr*, the gene contains a prokaryote-like promoter and ribosome binding site that permit its expression within the bacterium. The nature of the cytokinin hydroxylation reaction has not yet been determined, but *Agrobacterium* strains bearing the *tzs* locus hydroxylate iPMP and secrete ribosylzeatin and zeatin into the culture medium. Whereas its biochemical function has now been established, the biological role of this enzyme is not yet known, and the reason for its presence on nopaline Ti plasmids has yet to be determined.

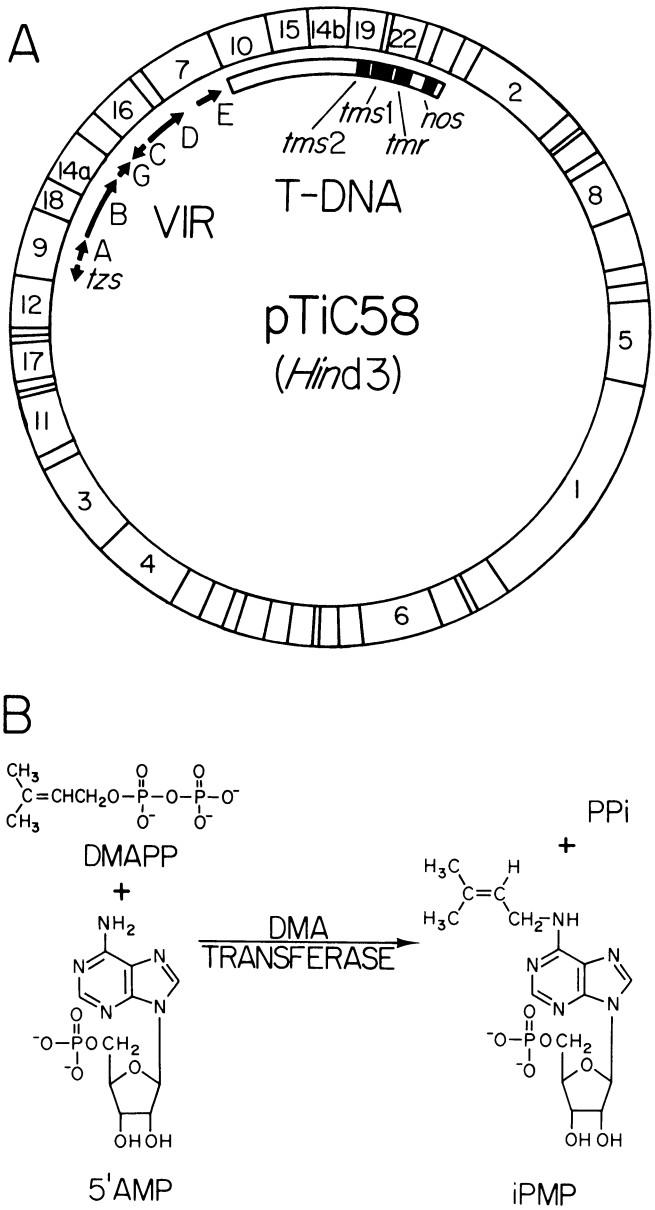


Fig. 1. A, Physical map of *A. tumefaciens* pTiC58 showing the location of the *tzs* gene, as well as the T-DNA (*tmr* = cytokinin biosynthetic gene; *tms*1 and *tms*2 = auxin biosynthetic genes) and VIR (*virA-virG* and *tzs*) regions. **B**, Dimethylallyl transferase (DMA transferase) catalyzed reaction. Abbreviations: DMAPP, dimethylallyl pyrophosphate; iPMP, isopentenyladenosine phosphate.

We now demonstrate that expression of *tzs* (but not *tmr*) is induced by several plant-derived phenolics in the same manner as the inducible virulence genes. DMA transferase activity and the secretion of zeatin are both increased more than 100-fold by addition of AS to the culture medium. As recently reported for the induction of several *Agrobacterium* virulence functions (Stachel and Zambryski 1986b), the activation of *tzs* expression requires only the presence of intact *virA* and *virG* loci. A preliminary account of this work has been reported (Morris 1988).

MATERIALS AND METHODS

Bacterial strains and plasmids. For analysis of cytokinins in culture filtrates, all strains were routinely cultured at 28° C (37° C for *E. coli*) in MSSP medium (Stachel *et al.* 1985) (1× Murashige and Skoog salts, 3% sucrose, 0.018% K_2HPO_4, 0.01% myo-inositol, 0.0001% biotin, 12.5 mM sodium phosphate, pH 5.5) supplemented with 10% (v/v) Luria-Bertani media. Acetosyringone was dissolved in dimethylsulfoxide and added to the media immediately before inoculation with 0.015 volume of the appropriate overnight culture. *A. tumefaciens* strains NT1, T37, A6, and Ach5 were obtained from E. Nester; RR5 and CG1C from L. Moore; and C58 from M.-D. Chilton.

Cosmids pTZ110, pTZ112, and pTZ134 were selected from a *Hind*III library prepared from pTiC58 in the vector pVK102 and maintained in *E. coli* HB101 (Regier and Morris 1982). Plasmid pTZ520 is a *tzs*-bearing plasmid that was constructed by insertion of a 1.4-kb *Hind*III-*Bam*HI fragment from pTZ120 (Beaty *et al.* 1986) into *Hind*III-*Bgl*II-digested pGA482 (An 1986), which contains the wide host range origin of replication, *oriV*, permitting it to be stably maintained in both *E. coli* and *A. tumefaciens*. The pGA482 vector was also used to prepare pTZ24, a plasmid that bears just *tzs*, *virA*, and *virG*. The details of this construction are shown in Figure 2.

By using the triparental mating procedure of Ditta *et al.* (1980) *tzs*-bearing cosmid and plasmid vectors were conjugally transferred into *A. tumefaciens* strain NT1 (a heat-cured Ti plasmidless derivative of strain C58). Plasmid isolations from *E. coli* were by the method of Birnboim and Doly (1979) and from *A. tumefaciens* by the rapid alkaline-phenol method of Ebert *et al.* (1987).

Cytokinin assays. The combination of zeatin and ribosylzeatin present in culture filtrates was determined either by radioimmunoassay (MacDonald and Morris 1985) or by ELISA (A. Battaille, personal communication). At the concentrations used in these experiments, none of the phenolics interfered with the assays.

Determination of DMA transferase activity in cellfree lysates. Cells from 5 ml of culture were centrifuged at 3,000 × *g* for 5 min. The cell pellet was resuspended in 1 ml of Buffer A (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, 20 mM $MgCl_2$, 10 mM 2-mercaptoethanol) and incubated for 15 min with lysozyme (1 mg/ml). The cells were then disrupted by four 30-sec pulses by using a Heat Systems-Ultrasonics sonicator. The lysates were centrifuged to remove cell debris and then dialyzed for 2 hr against 700 ml of Buffer A supplemented with 10% glycerol, four times.

Enzymatic activity was estimated by measurement of incorporation of labeled 5'-AMP into iPMP in the presence of DMAPP. The reaction mixture contained (in 100 μl): 1×

Buffer A; DMAPP, 0.4 μmol; [^{3}H]5′-AMP, 750 pmol, 2 μCi; unlabeled 5′-AMP, 500 μmol. After the addition of enzyme, the mixture was incubated for 1 hr at 25° C; the reaction was stopped by incubation at 65° C for 10 min, carrier iPA (100 ng) was added, and the nucleotides were dephosphorylated by incubating for 30 min at 37° C with 1 mg of alkaline phosphatase. Cytokinins were extracted into 100 μl of ethyl acetate and fractionated by thin-layer chromatography on Baker-flex silica gel IB-F plates developed with n-butanol:0.14 N NH_4OH:H_2O (6:1:2). Due to the presence of nucleotidase activity in crude bacterial extracts, radioactivity was present in both isopentenyladenine (iP) and isopentenyladenosine (iPA). The combined radioactivity was used as a measure of DMA transferase activity.

RESULTS

Induction of cytokinin biosynthesis in *A. tumefaciens* by acetosyringone. The close proximity of the *tzs* gene to the virulence region of nopaline Ti plasmids (Fig. 1A) led us to examine whether phenolics induce *tzs*-encoded DMA transferase activity and whether such induction would be reflected by changes in the secretion of zeatin. As shown in Figure 3A, addition of AS to early log phase cultures of the virulent *A. tumefaciens* strain C58 did indeed cause massive elevation in zeatin secretion. The concentration of zeatin rose to 35 μM, an increase of more than 100-fold above the level in untreated cultures in which zeatin concentrations of 0.05–0.2 μM were found routinely. Induction of cytokinin biosynthesis was evident at AS concentrations as low as 0.5 μM, but was greatest between 1 and 2 mM. When AS concentrations greater than 2 mM were added to the culture medium, cell growth was inhibited and a concurrent decline in cytokinin production was observed.

Altering the pH of the growth medium had a dramatic effect on the induction efficiency. In the presence of 200 μM of AS, the zeatin concentration was maximal at pH 5.0, but was significantly decreased at pH 4.5 and 6.0 (Fig. 3B). Zeatin production in uninduced cultures was also maximal at pH 5.0, but the levels were approximately 100-fold lower than those observed in the presence of AS (Fig. 3C).

Interestingly, AS induction of zeatin synthesis was only significant after the cultures had entered the late logarithmic phase of growth. Both DMA transferase activity and zeatin secretion increased very slowly for approximately 12 hr and then increased rapidly during the subsequent 36 hr (Fig. 4A). In contrast, cultures grown without AS showed a small increase in DMA transferase activity during the initial 24-hr period and then declined slowly thereafter. The zeatin level in untreated cultures remained low over the entire time course (data not shown). After 48 hr both DMA transferase and zeatin levels in induced cultures decreased slowly as the number of viable cells declined.

The effect of AS on the pattern of cellular protein expression was determined at each time point by

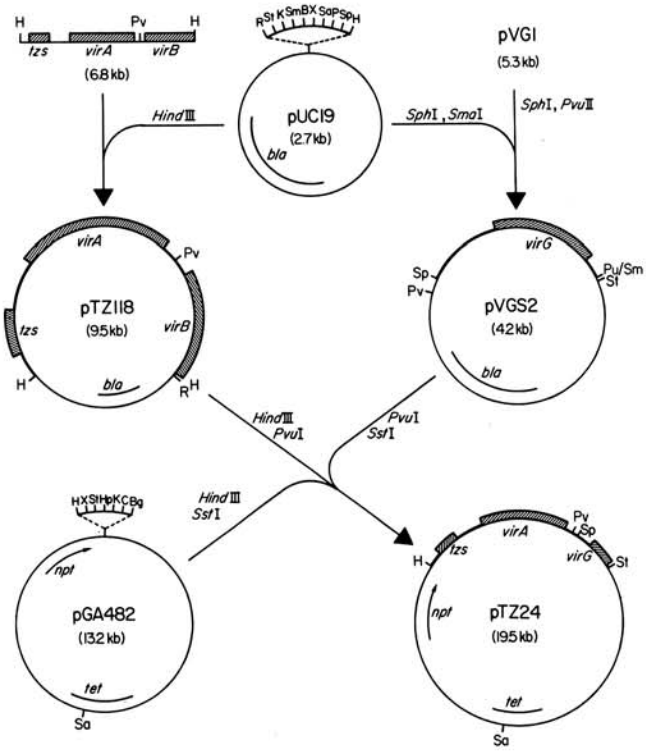


Fig. 2. Construction of plasmid pTZ24. The *tzs* and *virA* loci, together with the 5′ region of the *virB* locus, were isolated on *Hin*dIII fragment 9 of the C58 Ti plasmid and inserted into the *Hin*dIII site of pUC19 as shown, giving pTZ118. Digestion of pTZ118 with *Hin*dIII and *Pvu*I yielded a 4.7-kb fragment containing *tzs* and *virA*, but lacking the 5′ region from the *virB* loci. *VirG* was isolated from plasmid pVG1 (Powell *et al.* 1987) on a 1.4-kb *Sph*I/*Pvu*II fragment and cloned into pUC19 using the *Sph*I and *Sma*I sites present within the polylinker. The resulting construct, pVGS2, was digested with *Sst*I and *Pvu*I, yielding a 1.5-kb fragment bearing the entire *virG* locus. The two fragments isolated from pTZ118 and pVGS2 were then ligated to *Hin*dIII/*Sst*I-digested pGA482, a wide host range cloning vector. The resulting plasmid, pTZ24, was cloned into *E. coli* HB101 and then conjugally transferred to *A. tumefaciens* strain NT1. Restriction sites are denoted as follows: *Bam*HI, B; *Bgl*II, Bg; *Cla*I, C; *Eco*RI, R; *Hin*dIII, H; *Hpa*I, Hp; *Kpn*I, K; *Pst*I, P; *Pvu*I, Pv; *Pvu*II, Pu; *Sal*I, Sa; *Sma*I, Sm; *Sst*I, St; *Xba*I, X. *Tet*, *bla*, and *npt* denote tetracycline, ampicillin, and kanamycin resistance markers, respectively.

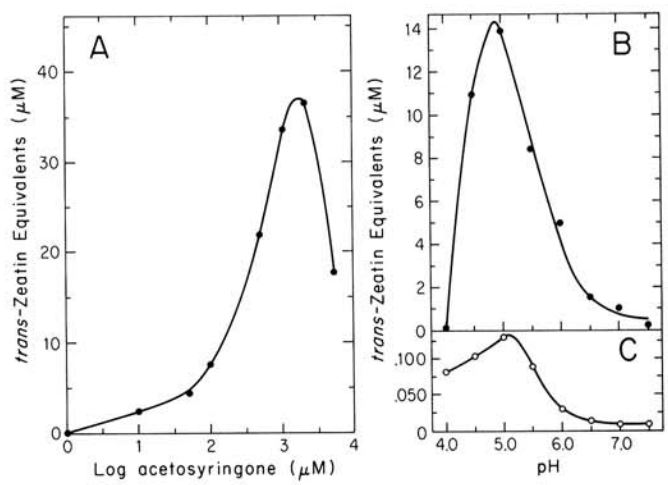


Fig. 3. A, Induction of zeatin secretion from *A. tumefaciens* C58 by acetosyringone (AS). AS was added to modified MSSP medium immediately before inoculation from an overnight culture. After 24 hr (approximately 16 hr after reaching mid log stage), the cells were pelleted at 15,000 × *g*. Ribosylzeatin and zeatin present in the culture filtrate were determined by radioimmunoassay. **B** and **C**, Effect of pH of the culture medium on cytokinin production. The pH of the MSSP medium was adjusted by supplementing the MSSP media with 125 mM phosphate buffer at the desired pH. The upper graph (**B**) shows zeatin levels measured in C58 cultures at pH 4.0–7.5 (AS = 200 μM). Open circles in graph C represent zeatin levels over the same pH range, but in the absence of AS.

polyacrylamide gel electrophoresis. Addition of AS to the growth media caused a number of new protein bands to appear, including a 28-kD protein that migrated at the position expected for DMA transferase (Fig. 4B). These data clearly show that the induction of DMA transferase activity, zeatin levels, and several proteins occurs only after a significant lag period lasting 6–12 hr.

Effect of other phenolics on cytokinin biosynthesis. The effect of several phenolics on zeatin levels is shown in Table 1. Of more than 20 compounds tested, most of which are structural analogs, AS was found to be the best inducer of cytokinin biosynthesis. Substitution of the acetyl group with either an acrylic group (sinapinic acid), or an aldehyde group (syringaldehyde) resulted in induction at 50% and 10%, respectively, of the level observed with AS. Loss of the 3′-methoxy group (acetovanillone) caused attenuation to 3% of the AS-induced level, whereas loss of both methoxy groups (4-hydroxyacetophenone) completely eliminated induction. Likewise, loss of the hydroxyl group (3,5-dimethoxyacetophenone) also abolished induction. Other structurally related compounds tested but found not to induce zeatin synthesis included chalcone, quercetin, fisetin, apigenin, naringenin, and coumesterol.

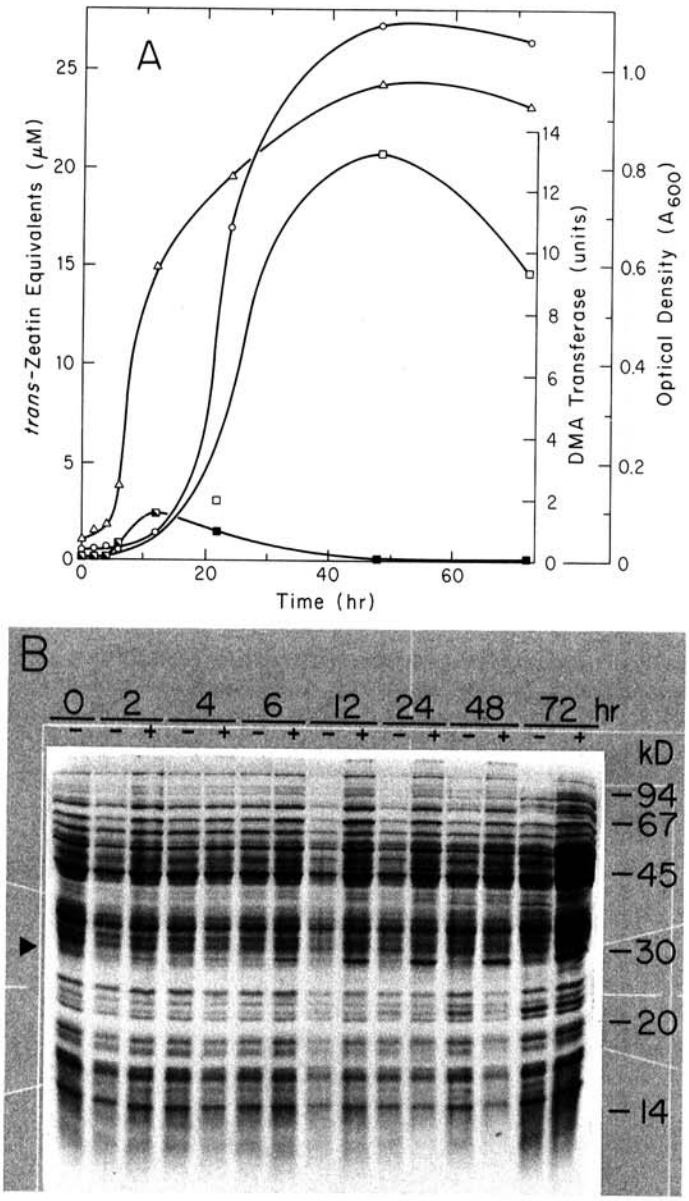


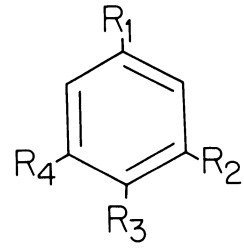
Fig. 4. A, Time course of AS-induced DMA transferase activity and zeatin levels in *A. tumefaciens* C58. Cells were induced with 500 μM AS, and aliquots were removed at specified time points for determination of DMA transferase activity and zeatin levels. Triangles = OD_{600}; open circles = [trans-zeatin]; open squares = DMA transferase activity; closed squares = DMA transferase activity in cells grown in the absence of AS. **B**, SDS-PAGE of total cellular proteins. At the times indicated along the top, cells grown in the presence (+) or absence (−) of AS were harvested by centrifugation, and samples corresponding to 1.2 to 1.4 × 10^8 cells were electrophoresed on a 15% polyacrylamide gel according to Laemmli (1970). The arrow indicates the position of an inducible 28-kD protein migrating at the position that would be expected for *tzs*-encoded DMA transferase. The gel was stained with Coomassie Brilliant Blue.

Induction of cytokinin biosynthesis is dependent on Ti plasmid-encoded factors. Because all *A. tumefaciens* strains examined contain *tmr*, but only nopaline strains contain *tzs*, it was essential to establish which of these genes was responsible for the observed increase in DMA transferase activity. Strain NT1, which contains no Ti plasmid, did not secrete zeatin at significant levels either in the presence or absence of AS. Strains A6, Ach5, and CG1C have octopine type Ti plasmids that contain only the *tmr* gene. All failed to secrete zeatin even when grown in the presence of AS (Table 2). However, all strains with a *tzs* gene, including the nopaline strains C58, T37, and RR5, secreted significant levels of zeatin. When these strains were grown with 100 μM of AS, zeatin levels were induced from 20- to 100-fold. Variations in both the basal and AS-induced levels of ribosylzeatin equivalents may reflect differences in cell growth rate and viability, DMA transferase specific activity, or intracellular substrate levels and availability. Substitution of the *tzs* gene with a kanamycin-resistance marker in the C58 Ti plasmid, by homologous recombination (details of this construction will be published elsewhere), resulted in the complete loss of zeatin synthesis and secretion, even upon induction with AS (data not shown), providing further evidence that *tzs*-encoded DMA transferase activity is responsible for AS-induced zeatin production.

If *tzs* and the inducible virulence loci are both controlled by the same factors, then *virA* and *virG* should be required for AS-induced *tzs* expression. To determine whether this was true, *tzs* was inserted, along with specific *vir* loci, into wide host range vectors and transferred into strain NT1. These strains were then examined for their ability to synthesize induced levels of zeatin when grown in the presence of AS. In the first experiment the *tzs* gene was inserted alone into the wide host range cloning vector pGA482, and the resulting plasmid, pTZ520, was then transferred into NT1. Although this strain secreted low levels of zeatin (0.05 μM), there was no increase in zeatin production in the presence of AS, indicating that inducible *tzs* expression is dependent on other genes encoded by the Ti plasmid. Identification of the other genes required for induction was achieved by transferring a set of cosmids, containing pTiC58 fragments, into strain NT1 and analyzing their ability to secrete zeatin in the presence of AS. As seen in Figure 5, only those cosmids containing *tzs*, *virA*, *virB*, and *virG* (i.e., pTZ112 and pTZ134) produced high levels of zeatin in the presence of AS. Cosmid pTZ110 (Beaty *et al.* 1986), which contains *tzs*, *virA*, and the 5′ region of *virB*, synthesized a low level of zeatin, but was not

Table 1. Relative activation of zeatin synthesis in *A. tumefaciens* C58 by different phenolic compounds

Phenolic compound	R_1	R_2	R_3	R_4	Zeatin (nM)	Zeatin induction[a]
Acetosyringone	$-COCH_3$	$-OCH_3$	-OH	$-OCH_3$	2,937	100
Sinapinic acid	$-CH=CHCO_2H$	$-OCH_3$	-OH	$-OCH_3$	1,487	51
Syringaldehyde	-CHO	$-OCH_3$	-OH	$-OCH_3$	248	8.5
Acetovanillone	$-COCH_3$	$-OCH_3$	-OH	-H	79	2.7
Syringic acid	$-CO_2H$	$-OCH_3$	-OH	$-OCH_3$	29	1.0
3,5-dimethoxyacetophenone	$-COCH_3$	$-OCH_3$	-H	$-OCH_3$	9	0.3
4-hydroxyacetophenone	$-COCH_3$	-H	-OH	-H	4	0.1
None					27	1.0

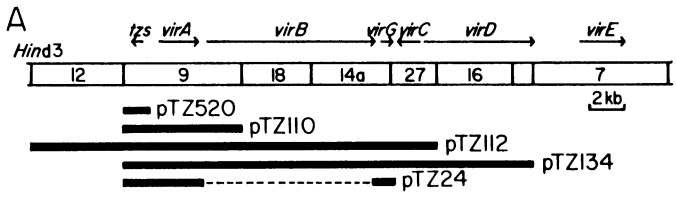


[a] Cultures were grown for 16 hr past mid log phase ($OD_{600} = 0.7$) with the indicated phenolic compound added to the medium to a concentration of 100 μM. Results for zeatin inducton are expressed as a percent of the level of zeatin produced with acetosyringone.

Table 2. Effect of acetosyringone on zeatin production by different *Agrobacterium* strains

Strain	Opine[a]	*tzs*[b]	*tmr*[b]	Ribosylzeatin equiv.[c] (μM) (−AS)	(+AS)
NT1	–	–	–	<.01	<.01
C58(pTiC58)	nop	+	+	.05	5.0
T37(pTiT37)	nop	+	+	.42	11.1
RR5(pTiRR5)	nop	+	+	.24	4.6
A6(pTiA6)	oct	–	+	<0.1	<.01
Ach5(pTiAch5)	oct	–	+	<.01	<.01
CG1C(pTiCG1C)	oct	–	+	.04	.09

[a] Nopaline Ti plasmid, nop; octopine, oct.
[b] The presence of the *tzs* and *tmr* loci was determined by Southern hybridization by using probes derived from pTiC58.
[c] Overnight cultures were inoculated into MSSP medium both in the presence and absence of 100 μM acetosyringone and incubated for 24 hr at 28° C. Zeatin and ribosylzeatin were determined by ELISA on aliquots from the culture filtrates. Ribosylzeatin was used as the standard.



B

Strain	*vir* loci	*tzs*	Zeatin (μM) (-AS)	(+AS)
NT1	none	-	<0.01	<0.01
NT1(pTZ520)	none	+	0.65	0.59
NT1(pTZ110)	*A,(B)*	+	1.38	1.20
NT1(pTZ112)	*A,B,G,C,(D)*	+	0.87	48.9
NT1(pTZ134)	*A,B,G,C,D*	+	0.61	44.3
NT1(pTZ24)	*A,G*	+	0.28	41.6

Fig. 5. Determination of Ti plasmid-encoded factors essential for *tzs* induction. **A**, The positions of *tzs* and the *vir* loci are shown above the *Hind*III restriction map of the virulence region of pTiC58. Segments of the *vir* region, designated by solid blocks, were inserted into wide host range vectors and conjugated into *A. tumefaciens* strain NT1 as described in the text. Plasmid designations are indicated to the right of the solid bars. **B**, Zeatin produced by each plasmid-bearing strain when grown in the absence or presence of 500 μM AS for 48 hr at 28° C. Zeatin present in culture filtrates was determined by ELISA.

induced by AS. These results indicate that *tzs* expression, like that of the other inducible virulence loci, is probably under the control of *virA* and *virG*. To prove this hypothesis, a wide host range plasmid pTZ24 containing only the *tzs*, *virA*, and *virG* genes from pTiC58 was constructed (Fig. 2). When pTZ24 was present in strain NT1, cytokinin biosynthesis was increased by AS to levels exceeding 40 μM (Fig. 5), confirming that *virA* and *virG* are the only Ti plasmid-encoded factors necessary for induction in *Agrobacterium*. Interestingly, neither the cosmids nor plasmid pTZ24 were inducible by AS when present in *E. coli*.

Comparison of the promoter regions of *tzs* and *E. coli ompC*. Regions of nucleotide sequence homology within the promoters of the *A. tumefaciens* C58 *tzs* gene (Beaty *et al.* 1986) and the osmotically regulated *E. coli ompC* gene (Mizuno and Mizushima 1986) suggest that both may be induced by the same, or a very similar, mechanism. A direct comparison of the promoter regions of *ompC* and *tzs* is shown in Figure 6. The *ompC* promoter includes three 10-bp direct repeats, (labeled a, b, and c), located just upstream from the −35 region, which is believed to be the OmpR regulatory protein binding site (Mizuno and Mizushima 1986). It is believed that the interaction of OmpR at this site induces, in an as yet unknown manner, transcription of *ompC*. The consensus sequence for these repeats is TGAAACATCT. The repeats are separated by 11 and 10 bp, respectively, which places them at approximately the same relative position, but one turn removed, along the DNA helix. The promoter region of the *tzs* gene contains two 10-bp sequences, TaAAACATCa and TGAAAaATCT, which share 80% and 90% identity, respectively, with the consensus sequence from *ompC* and are separated by 10 bp. The similarity between the *tzs* sequences and those of the *ompC* promoter suggests that they may share a common role and be directly involved in the regulation of *tzs* expression.

DISCUSSION

Cytokinin biosynthesis in nopaline strains of *Agrobacterium tumefaciens* is catalyzed by *tzs*-encoded DMA transferase activity and is regulated by acetosyringone and several other structurally related plant products. It is now evident that cytokinin production is regulated by the same system that controls the expression of the inducible virulence genes and activation of the T-DNA transfer process. We have also shown that inducible *tzs* expression exhibits many similarities with the expression of inducible genes from a number of prokaryotic systems.

Comparison of our results, which focus on the *tzs* gene, with the results of others who have examined the expression of *Agrobacterium* Ti plasmid-encoded virulence genes, indicates that both are induced by the *virA*/*virG* two-

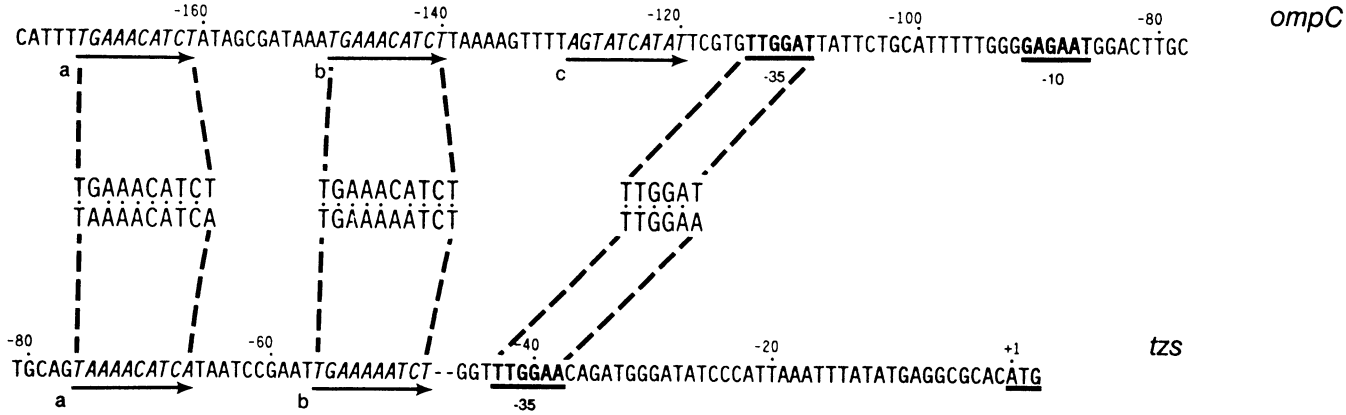


Fig. 6. Nucleotide sequence comparison of the promoter regions from the inducible *tzs* (Beaty *et al.* 1986) and *E. coli ompC* (Mizuno and Mizushima 1986) genes. The 10-bp direct repeats located in *ompC* (a, b, and c) and *tzs* (a and b) are italicized, and the asterisk above the *ompC* sequence indicates the transcription initiation site. The translational start codon (ATG) in *tzs* is double underlined. For numbering, the A's in both start codons are designated +1.

component regulatory system. Three factors lead to this conclusion.

First, the same chemical features that were found by Stachel *et al.* (1985) to be essential for the induction of *virB*, *C*, *D*, and *E* are also necessary for the induction of *tzs*. Acetosyringone proved to be the best inducer for both the *tzs* and *vir* genes, and other active compounds produced lower levels of induction. However, the relative level of induction of the *tzs* gene, caused by incubation of cells with each compound, was found to parallel the induction found for the virulence genes. Differences in the absolute levels of induction are likely caused by differences in cell-growth conditions and differences in the methods of measuring induction (i.e., we measured the metabolic product of DMA transferase activity, zeatin, synthesized *in vivo*, whereas Stachel *et al.* (1985) measured the enzymatic activity of β-galactosidase *in vitro*).

Second, induction of both *tzs* (this report) and *vir* gene expression (Stachel *et al.* 1987) is strictly dependent on environmental conditions, specifically, on the pH of the medium. *Agrobacterium* is a soil bacterium that thrives in soils of pH 5.5–6.0. It is possible that to activate the *tzs* induction system in *Agrobacterium*, the AS receptor must assume a conformation that is only found at this restricted pH. Alternatively, AS may not be the compound that is directly responsible for *tzs* and *vir* induction, but rather, may require conversion, possibly by bacterial enzymes active around pH 5.0, to the active signal molecule. It is interesting that zeatin production in uninduced cells exhibits the same pH response as in AS-induced cells, although at levels approximately 100-fold lower. This suggests that the induction process may be active, albeit at a very reduced level, even in the absence of a signal molecule.

Third, a factor common to both *tzs* and *vir* gene induction is a complete dependence on the presence of both the *virA* and *virG* gene products. Loss of either or both of these factors results, as we show here, in the complete loss of AS-inducible expression of *tzs* and, as shown by Stachel and Zambryski (1986b), loss of inducible expression of the virulence genes.

One of the most extensively investigated of the bacterial two-component regulatory systems is the *E. coli ompB* locus, which encodes two proteins, OmpR and EnvZ. These gene products are structurally quite similar to *Agrobacterium*-encoded VirG and VirA, respectively (Winans *et al.* 1986; Powell *et al.* 1987; Leroux *et al.* 1987; G. K. Powell, unpublished results), and probably function in a similar manner. The *ompR/envZ* gene products regulate expression of the outer membrane porin genes *ompC* and *ompF* in response to changes in environmental osmolarity. Transcription of the *ompC* gene is induced by increasing osmolarity and is believed to be mediated at the transcriptional level by binding of the OmpR protein to one or more of the 10-bp direct repeats that are located in the upstream region of the *ompC* promoter. These sequences have been found to be essential for osmotically-induced *ompC* synthesis (Mizuno and Mizushima 1986). The occurrence of two similar direct repeats in the promoter region of *tzs*, as well as highly conserved sequences in the promoter regions of *virB*, *virC*, *virD*, *virE*, and *virG* (the proposed 12-bp *vir*-box [Winans *et al.* 1987] includes six nucleotides from the 10-bp sequence plus an additional six nucleotides upstream) suggests that a common mechanism of induction may be shared between all of these systems. However, no direct evidence is currently available to demonstrate the involvement of these sequences in the induction of the virulence loci. A complete understanding of the molecular details of the *ompR/envZ* system could provide considerable insight into the processes controlling induction of *tzs* and the other *Agrobacterium* virulence loci.

The physiological role of DMA transferase catalyzed cytokinin accumulation and secretion has not yet been established and is complicated by the fact that *Agrobacterium* strains that lack a *tzs* gene (e.g., octopine strains) are still quite capable of infecting and transforming their respective plant hosts. Because of the direct association between the expression of *tzs* and *vir* genes, we believe that zeatin synthesis and secretion must play a crucial role in the virulence process in nopaline strains of *A. tumefaciens*. It is possible that the presence of cytokinins may have some effect on host range. Alternatively, if plant cells must be at a specific stage in their cell cycle to be susceptible to *Agrobacterium*-caused transformation, then cytokinins, by inducing cell division at wound sites, might provide the target for the invading bacteria. This hypothesis is interesting in that it can accommodate the fact that not all *Agrobacterium* strains contain *tzs*. One may suppose that the hosts of *tzs*-deficient strains effectively induce cell

division at wound sites even in the absence of externally supplied cytokinins, thereby presenting susceptible host cells. Discovery of the role(s) played by cytokinins in *A. tumefaciens*, as well as several other phytopathogenic bacteria in which they have been found, should aid in our understanding of the virulence process.

Note added in proof: Following acceptance of this manuscript for publication, we were informed of a recent report on the phenolic induction of cytokinin biosynthesis in *Agrobacterium* (M. C. John and R. M. Amasino 1988. J. Bacteriol. 170:790-795). Although we observed that induction of cytokinin synthesis exhibited a marked pH dependence, no such effect was found by John and Amasino. The reason for this difference is not clear.

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