The Tzs Protein and Exogenous Cytokinin Affect Virulence Gene Expression and Bacterial Growth of *Agrobacterium tumefaciens*

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ABSTRACT

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The soil phytopathogen *Agrobacterium tumefaciens* causes crown gall disease in a wide range of plant species. The neoplastic growth at the infection sites is caused by transferring, integrating, and expressing transfer DNA (T-DNA) from *A. tumefaciens* into plant cells. A *trans*-zeatin synthesizing (*tzs*) gene is located in the nopaline-type tumor-inducing plasmid and causes *trans*-zeatin production in *A. tumefaciens*. Similar to known virulence (*Vir*) proteins that are induced by the *vir* gene inducer acetosyringone (AS) at acidic pH 5.5, Tzs protein is highly

induced by AS under this growth condition but also constitutively expressed and moderately upregulated by AS at neutral pH 7.0. We found that the promoter activities and protein levels of several AS-induced *vir* genes increased in the *tzs* deletion mutant, a mutant with decreased tumorigenesis and transient transformation efficiencies, in *Arabidopsis* roots. During AS induction and infection of *Arabidopsis* roots, the *tzs* deletion mutant conferred impaired growth, which could be rescued by genetic complementation and supplementing exogenous cytokinin. Exogenous cytokinin also repressed *vir* promoter activities and Vir protein accumulation in both the wild-type and *tzs* mutant bacteria with AS induction. Thus, the *tzs* gene or its product, cytokinin, may be involved in regulating AS-induced *vir* gene expression and, therefore, affect bacterial growth and virulence during *A. tumefaciens* infection.

Agrobacterium tumefaciens, a plant pathogen residing in soil, can genetically transform many plant species and a few other eukaryotic species. *A. tumefaciens* causes crown gall diseases on plants, resulting in great economic losses in plant nurseries, stone fruit orchards, and vineyards. *A. tumefaciens* can persist in infested soils and is almost impossible to eliminate completely from soils. The transformation mechanism involves the transfer of a specific DNA molecule (transfer DNA [T-DNA]), processed from a large bacterial tumor-inducing (Ti) plasmid, through several biological membranes to the plant nucleus, where the DNA is integrated (17,33). The transfer of DNA from *A. tumefaciens* into a plant cell requires several virulence (*vir*) genes that reside on the Ti plasmid. The *vir* genes are induced by acidity, monosaccharides, phenolic compounds, and, especially, acetosyringone (AS), secreted from plant wounded sites (50).

The phenolics are sensed by the VirA/VirG two-component system and induce all *vir* gene expressions (11,12,33,50). Unlike phenolic enhancement, signaling pathway enhancement by sugars occurs through a chromosomally encoded glucose/galactose-binding protein, ChvE, which, in turn, interacts with VirA (11,33). VirD1 and VirD2 proteins together relax and excise the single-strand T-DNA from the Ti plasmid (16). VirC1 binds to the overdrive sequence and greatly enhances T-DNA border cleavage by the site-specific endonuclease (VirD1/VirD2) in *A. tumefaciens* (54,55). VirD2 protein attaches to the 5' end of the T-strand and serves as a pilot protein to guide the T-DNA from bacteria into the

plant cell (18). The export of the T-DNA and several Vir proteins requires the expression of the *virB1-11* and *virD4*, which form the type IV secretion system, including a transmembrane transporter and the filamentous pilus (T-pilus) (65). With the help of various plant proteins inside plant cells, the VirD2/T-DNA complex, coated with numerous molecules of VirE2, enters the plant nucleus and integrates into chromosome, finally expressing the transgene in the T-DNA region.

Plant hormone biosynthesis genes on the wild-type T-DNA include *iaaM*, *iaaH*, and *ipt*, which cause abnormal auxin and cytokinin production in infected plant cells and result in tumor growth. The *iaaM* gene encodes tryptophan 2-monooxygenase and converts tryptophan to indole 3-acetamide (52,58). The *iaaH* gene codes for indole 3-acetamide hydrolase and converts indole 3-acetamide to indoleacetic acid (IAA), one type of auxin (46,53). IAA acts as a signal indicating the plant transformation events by *A. tumefaciens* and also shuts down *vir* gene expression and inhibits bacterial growth by competing with AS for interaction with VirA (32). Other plant hormones, such as salicylic acid (SA), can inhibit *vir* gene expression, bacterial growth, bacterial attachment to plant cells, and virulence of *A. tumefaciens* (6,63). Additionally, 2-hydroxy-4,7-dimethoxybenzoxazin-3-one secreted from maize roots can inhibit *A. tumefaciens* *vir* induction and bacterial growth; therefore, these naturally existing inhibitors may participate in the plant defense response to *A. tumefaciens* (66).

The *ipt* gene codes for isopentenyl transferase, which utilizes dimethylallyl diphosphate or 4-hydroxy-3-methyl-2-(E)-butenyl diphosphate as the isopentenyl side-chain precursors and then converts to cytokinin isopentenyladenosine 5'-monophosphate or zeatin riboside 5'-monophosphate (1,2,7,9,42,43). *A. tumefaciens* contains another isopentenyl transferase-encoding gene, *trans*-zeatin synthesizing (*tzs*), which is homologous to the T-DNA-encoded *ipt* gene and located upstream of *virA* of the nopaline-type Ti plasmid (4,10,38). The *tzs* gene expression and *trans*-zeatin

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*The e-Xtra logo stands for "electronic extra" and indicates that the online version contains five supplementary table and one supplementary figure.

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secretion are both controlled by the VirA/VirG two-component regulatory system and are induced by AS in *A. tumefaciens* (6,13, 23,29,38,63). The *ipt* gene is under eukaryotic regulatory control and is involved in cytokinin synthesis in plant cells, whereas *tzs* displays a prokaryotic regulatory structure and participates in cytokinin synthesis in *A. tumefaciens*. Both *ipt* and *tzs* share sequence homology with other isopentenyl transferase genes in other prokaryotes such as *Pseudomonas savastanoi* pv. *savastanoi* and *Xanthomonas oryzae* pv. *oryzae* (3,30,39).

The Tzs protein localizes in the *A. tumefaciens* membrane and on the cell surface and interacts with VirB5 in yeast and in vitro (5,29). Furthermore, our previous study (20) demonstrated that the absence of Tzs protein expression and *trans*-zeatin secretion in the *tzs* frameshift (*tzs-fs*) mutant reduced stable and transient transformation efficiency in *Arabidopsis*. The addition of exogenous cytokinin during infection enhanced the transient transformation efficiency of *A. tumefaciens* on *Arabidopsis* roots, so that Tzs, by mediating *trans*-zeatin synthesis in *A. tumefaciens*, may affect the plant transformation process at early steps.

In this study, we aimed to elucidate further how Tzs or *trans*-zeatin participate during *A. tumefaciens* infection by investigating the effects of a *tzs* deletion mutant on *vir* gene expression, bacterial growth, and virulence. Similar to results obtained with the *tzs* frameshift mutant, the *tzs* deletion mutant reduced virulence in potato tuber discs and *Arabidopsis* roots. Interestingly, Vir protein accumulation and *vir* gene promoter activities were increased in the *tzs* deletion mutant during AS induction. The increase in AS-induced Vir protein level was associated with severity of impaired bacterial growth with the *tzs* deletion mutant under AS induction and infection. The presence of exogenous cytokinin decreased both *vir* gene promoter activity and Vir protein accumulation in *A. tumefaciens* during AS induction but increased bacterial growth during infection of *Arabidopsis* roots. The *tzs* gene or its cyto-

kinin product may contribute to *A. tumefaciens* virulence, likely via two major mechanisms that affect early stages of the transformation process: bacterial cell growth and *vir* gene expression.

MATERIALS AND METHODS

Plasmid construction. The bacterial strains, plasmids, and primer sequences are shown in Table 1 and Supplementary Table 1. Plasmid DNA isolation from bacterial cells and polymerase chain reaction (PCR) product purification involved use of the Plasmid Miniprep Purification and DNA Clean/Extraction kits (Hopegen Biotechnology Development Enterprise, Taipei). DNA cloning and PCR were as described (44). The *A. tumefaciens* *tzs* deletion mutant strain was generated by a double-crossover recombination with the suicide plasmid pJQ200KS (40). The plasmid pJQ200KS- Δ *tzs*::Sp^R, used to generate the *tzs* deletion mutant, was constructed by ligating the *Xma*I/*Bam*HI-digested Tzs1 PCR product (927-bp DNA fragment downstream of *tzs* coding region), the *Bam*HI-digested spectinomycin-resistant (Sp^R) gene cassette from the plasmid pGEMT-Sp (62), and the *Bam*HI/*Xba*I-digested Tzs2 PCR product (703-bp DNA fragment upstream of *tzs* coding region) into the *Xma*I/*Xba*I sites of the suicide vector pJQ200KS. *A. tumefaciens* cells grown overnight in 523 medium (24) with appropriate antibiotics were used to isolate genomic DNA with the Qiagen genomic DNA kit (Qiagen Inc., Valencia, CA). Tzs1 PCR product was amplified with use of the Advantage 2 PCR Enzyme System (BD Biosciences Clontech, Palo Alto, CA) with 1 μ g of genomic DNA as the template and primers Tzs1-*Xma*I-F and Tzs1-*Bam*HI-R. Tzs2 PCR product was amplified with the primers Tzs2-*Bam*HI-F and Tzs2-*Xba*I-R. The PCR amplification cycles were as follows unless indicated: 95°C for 2 min (1 cycle); 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min/per kb (35 cycles); and 72°C for 10 min (1 cycle).

TABLE 1. Bacterial strains and plasmids

Strains, plasmids	Relevant characteristics ^a	References, sources ^b
Strains		
<i>Agrobacterium tumefaciens</i>		
NT1RE	Rm ^R Em ^R , C58 cured of its pTiC58	60
NT1RE(pJK270)	Rm ^R Em ^R , Km ^R /Nm ^R , pJK270 is pTiC58Tra ^c with <i>Tn5</i> insertion in T-DNA region without affecting virulence	25
NT1RE(pJK270 Δ <i>tzs</i>)	Rm ^R Em ^R , Km ^R /Nm ^R , Sp ^R , pJK270 with Δ <i>tzs</i> ::Sp ^R	This study
<i>Escherichia coli</i>		
DH10B	Host for DNA cloning	Invitrogen
S-17	Host for conjugation	49
Plasmids		
pGEMT-Easy	Ap ^R , TA cloning vector	Promega
pGEMT-Sp	Ap ^R , Sp ^R , pGEMT-easy vector containing spectinomycin resistant gene cassette	62
pJQ200KS	Plasmid containing Gm ^R and SacB gene for selection of double crossover	40
pJQ200KS- Δ <i>tzs</i> ::Sp ^R	Gm ^R , pJQ200KS carrying Δ <i>tzs</i> ::Sp ^R	This study
pJQcom	Gm ^R , pJQ200KS carrying <i>ActC</i> locus	This study
pJQcom-Tzs	Gm ^R , pJQcom carrying <i>Tzs</i> gene	This study
pRU1156	Ap ^R , Tc ^R , a stable broad host range promoter-probe vector containing <i>gfpmut3.1</i> and <i>gusA</i>	26
pRUvirAp-gfp	Ap ^R , Tc ^R , the promoter region of <i>virA</i> is cloned into pRU1156 in the upstream of promoter-less <i>gfp</i> to generate <i>virA</i> _{pro} :: <i>gfp</i> transcriptional fusion	This study
pRUvirBp-gfp	Ap ^R , Tc ^R , the promoter region of <i>virB</i> is cloned into pRU1156 in the upstream of promoter-less <i>gfp</i> to generate <i>virB</i> _{pro} :: <i>gfp</i> transcriptional fusion	This study
pRUvirCp-gfp	Ap ^R , Tc ^R , the promoter region of <i>virC</i> is cloned into pRU1156 in the upstream of promoter-less <i>gfp</i> to generate <i>virC</i> _{pro} :: <i>gfp</i> transcriptional fusion	This study
pRUvirDp-gfp	Ap ^R , Tc ^R , the promoter region of <i>virD</i> is cloned into pRU1156 in the upstream of promoter-less <i>gfp</i> to generate <i>virD</i> _{pro} :: <i>gfp</i> transcriptional fusion	This study
pRUvirEp-gfp	Ap ^R , Tc ^R , the promoter region of <i>virE</i> is cloned into pRU1156 in the upstream of promoter-less <i>gfp</i> to generate <i>virE</i> _{pro} :: <i>gfp</i> transcriptional fusion	This study
pRUtzs-gfp	Ap ^R , Tc ^R , the promoter region of <i>Tzs</i> is cloned into pRU1156 in the upstream of promoter-less <i>gfp</i> to generate <i>tzs</i> _{pro} :: <i>gfp</i> transcriptional fusion	This study
pAC01-virBp	Ap ^R , Tc ^R , the promoter region of <i>virB</i> is cloned into pAC01 in the upstream of promoter-less <i>lacZ</i> to generate <i>virB</i> _{pro} :: <i>lacZ</i> transcriptional fusion	27
pCambia 2201-Gm	Cm ^R , Gm ^R , a binary vector for plant transformation	20

^a Ap^R, Cm^R, Em^R, Gm^R, Km^R, Nm^R, Rm^R, Sp^R, and Tc^R = resistant to ampicillin, chloramphenicol, gentamicin, erythromycin, kanamycin, neomycin, rifampicin, spectinomycin, and tetracycline, respectively; *gfp* = green fluorescent protein.

^b Invitrogen = Invitrogen Corporation, Carlsbad, CA and Promega = Promega Corp., Madison, WI.

To generate the *A. tumefaciens tzs* genetic complementation strain at the chromosomal locus, the 1,009-bp genomic fragment of the *tzs* region was amplified by PCR and introduced upstream of the *actC* locus, which is not required for virulence, in the *A. tumefaciens* circular chromosome (31) by double-crossover recombination. We first generated the pJQcom plasmid to introduce the desired gene into this locus. The downstream region covering the partial *actC* locus was amplified by PCR with the primers comp-L-F and comp-L-R. The upstream region of the *actC* locus was amplified with the primers comp-R-F and comp-R-R. Both DNA fragments were cloned into the TA cloning vector pGEMT-Easy (Promega Corp., Madison, WI) and confirmed by sequencing. These two fragments were then cloned into the pJQ200KS plasmid to result in the pJQcom plasmid. The *tzs* PCR product, amplified with primers TZSexp-F and TZSexp-R, was digested and ligated into the *Bam*HI site of the pJQcom vector to create the pJQcom-Tzs plasmid.

To generate plasmids for promoter activity assays, the promoter regions of *virA*, *virB*, *virC*, *virD*, *virE*, and *tzs* were amplified by PCR with primers in Supplementary Table 1 from *A. tumefaciens* NTIRE(pJK270) cells, digested with *Spe*I and *Hind*III, and ligated into the same sites of the promoter-probe vector pRU1156 (26,57).

Generating the *A. tumefaciens tzs* deletion mutant and *tzs* genetic complementation strains. pJQ200KS- Δ *tzs*::Sp^R was used to generate the *A. tumefaciens tzs* deletion mutant strain with minor modifications (20,62). Overnight culture (5 μ l) (grown in Luria broth [LB] broth without antibiotics) of *Escherichia coli* S-17 containing pJQ200KS- Δ *tzs*::Sp^R and *A. tumefaciens* strain NTIRE(pJK270) were incubated at 28°C on LB agar overnight. The bacterial cells were then streaked out on LB agar containing erythromycin (Em, 50 μ g ml⁻¹), rifampicin (Rm, 50 μ g ml⁻¹) (MDBio, Inc., Taipei, Taiwan), gentamycin (Gm, 50 μ g ml⁻¹), and kanamycin (Km, 20 μ g ml⁻¹) for incubation at 28°C for 2 days to obtain the first crossover events. Several independent colonies were randomly selected and were independently grown in 5 ml of LB broth without antibiotics at 28°C overnight. Serial dilutions were plated onto 523 agar containing 5% sucrose, Em, Rm, and Km to grow at 28°C for 2 days. To generate the *tzs* deletion mutant strain, a colony that was Sp^R (200 μ g ml⁻¹), sucrose resistant, and Gm susceptible had undergone a second crossover to replace the respective wild-type gene with the Sp^R cassette. The *tzs* deletion mutant strain was subsequently confirmed by genomic colony PCR with the primers Tzs1-*Xma*I-F and Tzs2-*Xba*I-R and Southern hybridization (data not shown).

The plasmid pJQcom-Tzs was used to generate the genetic complementation strain of the *tzs* deletion mutant by integrating the complemented *tzs* gene on the circular chromosome. The *A. tumefaciens tzs* deletion mutant and *E. coli* S17 containing pJQcom-Tzs were grown in LB medium overnight. A DNA fragment including the *tzs* coding region and its native promoter was introduced into the upstream region of the *actC* locus of the *A. tumefaciens* circular chromosome of the *tzs* deletion mutant. Previous study demonstrated that destruction of *actC* locus by insertion of an Sp^R gene cassette does not affect *A. tumefaciens* virulence (31). The *A. tumefaciens* colony containing the *tzs* gene fragment in the upstream region of the *ActC* locus of the *A. tumefaciens* circular chromosome was selected for antibiotic resistance and confirmed by colony PCR with the primers comp-con-F and comp-con-R.

Immunoblotting analysis of proteins isolated from *A. tumefaciens* under different conditions. *A. tumefaciens* cells grown overnight in 523 medium with appropriate antibiotics were harvested (6,000 $\times$ g, 10 min) and resuspended in AB-MES media (pH 5.5 or 7) or 523 media (pH 5.5 or 7) without antibiotics with an absorbance at 600 nm (A_{600}) = 0.1. After growth at 28°C to mid-log phase for 6 h, the bacterial cultures were further cultivated at 19°C with 200 μ M AS (Sigma-Aldrich, St. Louis). Cells

were collected at 0, 16, 40, and 64 h, then protein was isolated as described (20). Bacterial protein extracts were resolved on 16.5% Tricine sodium dodecyl sulfate-polyacrylamide gels (MDBio, Inc., Taipei, Taiwan) (45) and transferred to Hybond ECL Nitrocellulose membrane (Amersham Biosciences, Uppsala, Sweden), which were incubated with primary antibodies against Tzs (1:10,000 dilution) (28), VirB2 (1:1,000) (47), VirB4 (1:10,000), VirB8 (1:10,000), VirB9 (1:5,000), VirB10 (1:5,000), VirB11 (1:10,000), VirD2 (1:22,000), VirE2 (1:20,000) (8), or neomycin phosphotransferase II (NptII) (1:50,000) (Sigma-Aldrich), then horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:50,000) (Chemicon International, Temecula, CA). The membranes were developed by use of a chemiluminescent detection method (PerkinElmer Life and Analytical Sciences, Boston) and underwent autoradiography. The protein bands were detected by the G:BOX ChemiXT gel documentation and analysis system (Syngene, Frederick, MD) and quantified by use of GeneTools (Syngene).

Determination of the virulence of the *A. tumefaciens tzs* deletion in *Arabidopsis* roots, potato tuber discs, and stems of other plant species. Quantitative tumorigenesis assays with potato tuber discs and *Arabidopsis* root transformation assays were as described (19,20,48,67), with minor modification. All *A. tumefaciens* strains were cultured in 523 media with appropriate antibiotics at 28°C and grown to A_{600} = 0.8 to 1.0. The bacterial cells were then washed with and resuspended in 0.9% sodium chloride at 10⁶ or 10⁸ CFU ml⁻¹ for potato tumorigenesis assays and 10⁷ or 10⁸ CFU ml⁻¹ for *Arabidopsis* transformation assays. For potato tumor assays, the discs were co-incubated with 10- μ l *A. tumefaciens* cultures for 2 days. Five weeks after infections, tumor numbers of each disc were scored. For each independent transformation assay, at least 60 potato discs were infected with each *A. tumefaciens* strain. For transient transformation assays, *Arabidopsis* root segments were infected with *A. tumefaciens* strains containing an additional binary vector pCAMBIA2201-Gm (20). At least 70 root segments from 10 different *Arabidopsis thaliana* plants (ecotype: Ws) were infected with 100 μ l of each *Agrobacterium tumefaciens* strain on Murishige and Skoog (MS) media with or without exogenous cytokinin in the media at 22 to 24°C for 2 days for each independent transformation assay. Seven days after infection, *Arabidopsis* root segments were stained with the 5-bromo-4-chloro-3-indolyl-b-D-glucuronic acid staining solution (20) for 1 day at 37°C to obtain transient transformation efficiencies. For *Arabidopsis* tumorigenesis assays, tumors on root segments were scored 5 weeks after infection. Tumor assays with other plant species were performed as described (20,36), with minor modifications. Bacteria cultures (100 μ l of 10⁹ CFU ml⁻¹) were injected with the use of a syringe into 3- to 4-week-old plant stems. At 5 to 7 weeks after inoculation, plants were collected and scored for tumors. With each tested *A. tumefaciens* strain, at least 15 different plants were infected for each independent transformation assay. The strain NTIRE (no Ti plasmid) was used as a negative control in each tumor assay. Data shown in the figures represent mean $\pm$ standard error (SE) from at least three independent experiments.

Promoter activity assays. The *virB* promoter activity was determined by β -galactosidase activity assay (31). The *virB* promoter *lacZ* transcriptional fusion plasmid pAC01-*virBp* (27) was introduced into wild-type and *tzs* deletion *A. tumefaciens* strains by conjugation. Overnight *A. tumefaciens* cultures were harvested and resuspended in AB-MES media (pH 5.5 or 7) or 523 media (pH 5.5 or 7) without antibiotics at A_{600} = 0.1. After growth at 28°C to A_{600} = 0.2 to 0.4, bacterial cultures were further cultivated at 19°C for 40 h with 200 μ M AS. Bacterial cells were then sampled to determine β -galactosidase activity as described (34). To determine the *virB* promoter activity on MS media, *A. tumefaciens* cultures were grown in 523 media (pH 7) with appropriate antibiotics at 28°C to A_{600} = 0.8 to 1, then washed and resus-

pended in 0.9% sodium chloride at 10^7 CFU ml⁻¹ and further cultured on MS medium containing 200 μ M AS with or without kinetin at 0.01 ppm (mg liter⁻¹) and 22 to 24°C up to 12 h. Bacterial cells were collected at 8 and 12 h for β -galactosidase activity assays.

The *virA*, *virB*, *virC*, *virD*, *virE*, and *tzs* promoter activity in the wild-type and the *tzs* deletion *A. tumefaciens* strains were determined by green fluorescence protein (GFP) activity assay, as described (57). *A. tumefaciens* cells containing pRUvirp-gfp or pRUtzsp-gfp plasmids were first cultured in AB-MES media (pH 5.5) at 28°C to $A_{600} = 0.2$ to 0.4 and further cultured in the same media containing AS at 19°C up to 8 h with or without kinetin at 0.01 ppm, then collected and normalized to $A_{600} = 0.2$ with 0.9% sodium chloride. A 200- μ l bacterial suspension was aliquoted into Nunc F96 MicroWell plates (Thermo Fisher Scientific, Pittsburgh, PA) and GFP fluorescence was measured with use of the PARADIGM Detection Platform (Beckman Coulter, Indianapolis, IN) at 535-nm emission and 485-nm excitation. The promoter activities shown in figures are expressed as fluorescence units relative to the increase in GFP fluorescence intensity per bacterium per milligram of protein.

Growth curve analyses of *A. tumefaciens* strains under various growth conditions. *A. tumefaciens* cells were first cultured in 523 medium with appropriate antibiotics and collected and resuspended in AB-MES media (pH 5.5 or 7) or 523 media (pH 5.5 or 7) without antibiotics at $A_{600} = 0.1$. After bacteria grew at 28°C up to $A_{600} = 0.2$ to 0.4, cultures were divided into two bottles and further incubated at 19°C with or without 200 μ M AS. At 0, 16, 40, and 64 h, cells were collected by centrifugation, serially diluted with 0.9% sodium chloride, and cultured on 523 agar plates with appropriate antibiotics to determine viable cell numbers. To determine the viable bacterial cell numbers during infection of *Arabidopsis* roots, bacterial cultures were first prepared according to *Arabidopsis* root transformation assays. A 100- μ l amount of bacterial culture in 0.9% sodium chloride at 10^7 CFU ml⁻¹ cell density was used to infect *Arabidopsis* roots on MS medium with or without kinetin at 0.01 ppm and 22 to 24°C up to 48 h. The viable cell numbers were determined at 0, 24, and 48 h during co-culture. The viable cell numbers for each time point shown in figures are expressed as CFU per milliliter and represent mean $\pm$ SE from at least three independent experiments.

RESULTS

Tzs is expressed in the absence of AS and upregulated by AS moderately at neutral pH and highly at acidic pH. To understand further the molecular mechanism of how Tzs protein contributes to *A. tumefaciens* virulence, we first monitored Tzs protein expression under various growth conditions by immunoblotting analysis with anti-Tzs antibody (5,28). Several environmental factors, including temperature, pH, monosaccharide, and phenolic compounds, affect *vir* gene expression in *A. tumefaciens*. To determine whether the regulation of *tzs* expression is similar to that of other *vir* genes, we tested whether factors other than the phenolic compound (AS), such as pH, could affect Tzs protein expression in wild-type *A. tumefaciens*.

We examined Tzs protein expression in the wild-type *A. tumefaciens* strain, NT1RE(pJK270), cultivated in minimal media (AB-MES media) at acidic pH (pH 5.5) at 19°C with or without AS. Immunoblotting analysis revealed Tzs protein expressed at constant levels from 16 to 64 h of cultivation without AS incubation; VirB2 proteins were not detectable without AS (Fig. 1A). However, Tzs protein levels were greatly increased after the addition of AS at 16 h and remained constant up to 64 h (Fig. 1A). VirB2 protein expression was induced by AS and increased with increasing incubation time (Fig. 1A). VirB2 protein was not detected in minimal media at neutral pH (pH 7) at 19°C, a condi-

tion known not to induce *virB* expression (23,41,64) (Fig. 1B). Interestingly, Tzs protein expression was constant without AS and was upregulated with AS (Fig. 1B), similar to what was observed with acidic minimal medium (Fig. 1A).

We also checked Tzs protein expression in rich media (523 media) at pH 5.5 or 7 to understand whether the Tzs protein expression pattern was similar to that of other Vir proteins, such as VirB2. Tzs protein level was constant in the absence of AS and was induced by AS during 64 h in rich media with acidic or neutral pH at 19°C (Fig. 1C and D). VirB2 protein expression was induced by AS at a later time point, 64 h after the addition of AS, in the acidic rich media (Fig. 1C). In contrast, VirB2 protein expression was not detected in rich media at neutral pH even after adding AS (Fig. 1D), which is consistent with previous studies (41).

We used gel documentation analysis to quantify the relative Tzs protein amounts under different conditions. Overnight wild-type *A. tumefaciens* cultures grown in 523 media (pH 7) were used for further culture in AB-MES media (pH 5.5 or 7) or 523 media (pH 5.5 or 7) without antibiotics. Therefore, we normalized the relative Tzs protein level, in different media with or without AS at 4 time points, to Tzs protein level at 0 h in 523 media (pH 7) (Fig. 1D). Relative Tzs protein level was increased >60-fold in AB-MES media and 523 media at acidic pH (pH 5.5) with AS induction (Fig. 1E). When bacteria were grown in AB-MES media and 523 media at neutral pH, the relative Tzs protein level was almost 10-fold increased in the presence of AS (Fig. 1E). The relative Tzs protein level remained low in media containing no AS (Fig. 1A to D). Thus, Tzs is constitutively expressed and is upregulated by AS in rich (523) or minimal (AB-MES) media. In addition, the Tzs protein level may increase further with acidity. Interestingly, Tzs protein level was upregulated by AS in rich or minimal media at neutral pH when no Vir proteins were induced; therefore, Tzs protein expression is upregulated by AS under acidic conditions and regulated independent of pH and AS, which differs from stringent regulation of VirB protein expression by AS in acidic medium (Fig. 1A to D).

Absence of Tzs protein in the *tzs* deletion mutant reduced tumorigenesis and transient transformation efficiency in *Arabidopsis* roots. To determine the role of the Tzs protein in *A. tumefaciens*, we generated the *tzs* deletion mutant, Δ *tzs*, in the nopaline-type virulent strain NT1RE(pJK270). No Tzs protein was detected in the *tzs* deletion mutant strain when grown in the acidic AB-MES media at 19°C after AS treatment for 40 h (Fig. 2A). In addition, we detected AS-induced VirB2 and VirD2 protein level at 40 h; therefore, AS induction was successful. Because of the presence of the kanamycin-resistant gene in the Ti-plasmid (pJK270), we performed immunoblotting analyses with the anti-NptII antibody to demonstrate equal loading in each protein sample (Fig. 2A). High-performance liquid chromatography detected no *trans*-zeatin in the *tzs* deletion mutant under similar induction conditions (data not shown).

We then carried out quantitative tumor assays using potato (*Solanum tuberosum*) tuber discs and *Arabidopsis* roots to examine whether the absence of the Tzs protein affects *tzs* deletion-mutant virulence. The *tzs* deletion mutant showed >40 and 70% reduction in tumor-formation efficiency of potato and *Arabidopsis*, respectively, compared with the wild type (Fig. 2B and C). Previous tumor assay results of the *tzs* frame-shift mutant (20) displayed different tumor formation efficiencies compared with the wild-type strain on an additional six plant species, including white radish (*Raphanus sativus* cv. Rosy), pai-tsai (*Brassica rapa* L. R. Chinensis Group), loose-leaf lettuce (*Lactuca sativa* L. var. *crispa*), carnation (*Dianthus caryophyllus* L.), cowpea (*Vigna unguiculata* 'Green Pod' Kaoshiung), and green pepper (*Capsicum annuum* 'Vega'). Therefore, we tested these six plant species with the *tzs* deletion mutant. As compared with the wild type, the *tzs* deletion mutant showed reduced tumor-formation efficiency

by ≈ 30 to 50% in stems of white radish, pai-tsai, loose-leaf lettuce, and carnation (Supplementary Figure 1A). In contrast, the *tzs* deletion mutant caused 2.2- to 2.5-fold increase in tumor-formation efficiency on the stems of cowpea and green pepper as compared with the wild type (Supplementary Figure 1B).

Additionally, we performed quantitative transient transformation assays with *A. tumefaciens* strains containing the T-DNA binary vector pCAMBIA2201-Gm (20) on *Arabidopsis* roots. *Arabidopsis* roots infected with the *tzs* deletion mutant showed >70 to 80% reduction in transient transformation efficiency as compared with the wild type (Fig. 2D and E). To determine whether the lower stable and transient transformation efficiency of the *tzs* deletion mutant resulted from loss of Tzs protein, we performed genetic complementation testing. Tzs protein accumulation, tumorigenesis, and transient transformation efficiency were restored to the wild-type level in the *tzs*-complemented strain (Fig. 2A, B, and D); therefore, loss of Tzs protein expression is responsible for the *tzs* deletion-mutant phenotype.

Tzs protein possesses isopentenyl transferase activity, which leads to the production of cytokinin in *A. tumefaciens* (38). Therefore, the reduced virulence phenotypes of the *tzs* deletion mutant may be due to lack of cytokinin production in *A. tumefaciens*. Chemical complementation tests were performed to determine whether the addition of cytokinin during infection could complement the *tzs* deletion mutant phenotypes. Various concentrations of the cytokinin kinetin (0.01, 0.3, 0.5, 1, 2.5, 5, and 8 ppm [mg

liter⁻¹]) were added in the MS media during 2-day co-cultivation of *Arabidopsis* in stable and transient transformation assays. With increased concentrations of kinetin from 0.01 ppm (0.05 μ M) to 8 ppm (37.2 μ M) in the media during infection, both stable and transient transformation rates of the *tzs* deletion mutant were increased >twofold as compared without kinetin (Fig. 2C and 2E). In contrast, the addition of kinetin did not significantly increase either stable or transient transformation rates with 10^7 CFU ml⁻¹ wild-type bacterial cells used to infect *Arabidopsis* roots (Fig. 2C and E). Thus, the lower transformation efficiency of the *tzs* deletion mutant may have resulted from the absence of secreted cytokinin during infection, which is consistent with previous results from the *tzs* frame-shift mutant (20). These data also strongly support the idea that Tzs or *trans*-zeatin play a role in the early steps of *Agrobacterium*-mediated plant transformation.

The absence of Tzs protein and exogenous cytokinin affected *Agrobacterium* Vir protein accumulation and *vir* promoter activities. We wondered which early steps of Tzs or *trans*-zeatin activity were involved in *Agrobacterium*-plant interactions. AS-induced VirB2 and VirD2 protein levels were greater in the *tzs* deletion mutant than the wild type (Fig. 2A). Therefore, we examined the accumulation of other Vir proteins, including VirE2, VirB4, VirB9, VirB8, VirB10, and VirB11, in both the wild type and *tzs* deletion mutant in acidic AB-MES media at 19°C, 16 and 40 h after the addition of AS. With increased AS treatment time, all Vir protein levels increased in both the wild type and *tzs* de-

Fig. 1. Tzs protein is continuously expressed and upregulated by acetosyringone (AS) treatments under various growth conditions. The wild-type *Agrobacterium tumefaciens* strain NTIRE(pJK270) was grown in **A**, acidic AB-MES media (pH 5.5); **B**, neutral AB-MES media (pH 7); **C**, acidic 523 media (pH 5.5); or **D**, neutral 523 media (pH 7) in the absence (–) or presence (+) of 200 μ M AS at 19°C for 0, 16, 40, and 64 h. Total bacterial proteins were analyzed by 16.5% Tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis, then immunoblotting with antibodies against Tzs, VirB2, and neomycin phosphotransferase II (NptII). The molecular mass marker is indicated on the left (in kilodaltons) and the positions of Tzs, VirB2, and NptII are indicated on the right. **E**, Quantitative analyses of the Tzs protein expression. AS-induced Tzs protein levels with four kinds of media were normalized to Tzs protein level at 0 h in 523 media (pH 7).

letion mutant (Fig. 3A). In addition to VirB2 and VirD2, AS-induced VirE2, VirB4, B8, B9, B10, and B11 protein levels were higher in the *tzs* deletion mutant than the wild type at both 16 and 40 h after AS induction (Fig. 3A). In contrast, the internal control NptII protein level remained constant in both bacterial strains during AS treatment, which suggests that the deletion of *tzs* affected mainly AS-induced Vir protein level (Figs. 2A and 3A). In addition, in the *tzs*-complemented strain, AS-induced VirB2 and VirD2 protein levels were reduced to the wild-type level (Fig. 2A), which suggests that absence of the Tzs protein increased several Vir protein levels with AS induction. As well, AS-induced Vir protein levels were higher in the *tzs* deletion mutant than the wild type at earlier times (4 and 8 h) in acidic AB-MES media at 25°C (Fig. 3B). The levels of VirD2 and several VirB proteins were higher in the *tzs* deletion mutant than the wild type at both 16 and 40 h after the addition of AS in AB-MES media (pH 7) or 523 media at neutral or acidic pH (Fig. 3C to E). In summary, the absence of the Tzs protein resulted in increased AS-induced Vir protein level in *A. tumefaciens* grown in minimal (AB-MES) or rich (523) media at acidic or neutral pH.

Supplementation with exogenous cytokinin during infection restored the transformation efficiency of the *tzs* deletion mutant (Fig. 2B to E). Thus, we examined whether the addition of kinetin

in the acidic AB-MES media with AS treatment up to 16 h at 19°C could affect Vir protein level in both the wild type and *tzs* deletion mutant. Immunoblotting revealed decreased AS-induced VirD2, VirB2, B4, B10, B11, and Tzs protein levels but protein levels of NptII remained constant in the presence of kinetin at 0.01 ppm (0.05 μ M) in the wild-type strain (Fig. 3F). Similar results were observed in the *tzs* deletion mutant after the addition of kinetin during AS induction (Fig. 3F). Therefore, AS-induced Vir protein level in the *tzs* deletion mutant may be caused by lack of secreted cytokinin during induction. Additionally, Tzs, secreted cytokinin, or both may affect Vir protein levels in *A. tumefaciens* during AS induction.

To determine whether the increase in Vir protein level is regulated at the transcriptional level, we first monitored the *virB* promoter activity by transcriptional fusion of each promoter to a *lacZ* reporter gene on the promoter probe plasmid pAC01 and expressed it in the wild type or *tzs* deletion mutant. Although *virB* promoter activities were upregulated by AS in both the wild type and *tzs* deletion mutant when grown in acidic AB-MES media at 19°C for up to 40 h, *virB* promoter activities were induced earlier and were 1.5- to 30-fold higher in the *tzs* deletion mutant than the wild type (Fig. 4A). As well, *virB* promoter activity was restored to the wild-type level in the *tzs* complemented strain (Fig. 4A).

Fig. 2. Absence of Tzs protein in the *tzs* deletion mutant decreases tumorigenesis and transient transformation efficiency in *Arabidopsis* roots. **A**, Immunoblotting analysis of Tzs, VirB2, VirD2, and neomycin phosphotransferase II (NptII) protein levels in the wild type (WT) *Agrobacterium tumefaciens* strain (NTIRE[pJK270]), *tzs* deletion mutant (Δ *tzs*), and *tzs*-complemented (Δ *tzs* + *tzs*) strains grown in acidic AB-MES media with 200 μ M acetosyringone (AS) at 19°C for 40 h. Equal amounts of protein were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis. NptII antibody was a loading control. **B**, Quantitative tumor assay results of the WT, Δ *tzs* mutant, and *tzs*-complemented (Δ *tzs* + *tzs*) strains in potato tumor assays. Potato tuber discs were infected with 10^6 or 10^8 CFU ml^{-1} bacterial concentrations as indicated. Approximately 20 to 30 tumors per disc were scored 5 weeks after infection with the WT strain. The tumorigenesis efficiency of the WT in B and D was set to 100% and that of the mutant is relative to that of the WT strain. Data are mean $\pm$ standard error from at least three independent experiments. Asterisk (*) indicates $P < 0.05$ between the WT and Δ *tzs* mutant strains by analysis of variance (ANOVA) or pairwise Student's *t* test. **C**, Chemical complementation tests of tumor assays with the WT (NTIRE[pJK270]) and the Δ *tzs* mutant. Root segments from 3- to 4-week-old *Arabidopsis thaliana* WT plants were infected with 10^7 CFU ml^{-1} concentrations of the WT and Δ *tzs* mutant on Murashige and Skoog (MS) media with or without different kinetin concentrations for 2 days. The letter 'a' above each bar represents statistically significant differences between with and without exogenous cytokinin application for the same strain by ANOVA or pairwise Student's *t* test. **D**, Quantitative transient transformation assays of the WT, Δ *tzs* mutant, and *tzs*-complemented (Δ *tzs* + *tzs*) strains in *Arabidopsis* roots assays. *Arabidopsis* roots were infected with 10^7 or 10^8 CFU ml^{-1} bacterial concentrations as indicated. The transformation efficiency of root segments infected with the WT, with ≈ 40 to 60% of roots showing β -glucuronidase (GUS) activity in each experiment, was set to 100% and that of the mutant is shown relative to that of the WT. **E**, Chemical complementation tests of transient transformation assays with the WT and Δ *tzs* mutant. *Arabidopsis* root segments were infected with 10^7 CFU ml^{-1} concentrations of the WT and Δ *tzs* mutant on MS medium with or without different kinetin concentrations for 2 days. Root segments were stained with 5-bromo-4-chloro-3-indolyl- β -D-glucuronic acid staining solution 7 days after infection.

Thus, higher *virB* promoter activity in the *tzs* deletion mutant may be due to loss of function of the Tzs protein.

Because the levels of several VirB proteins were increased in the *tzs* deletion mutant in AB-MES media (pH 5.5 or 7) or 523 media (pH 5.5 or 7) with AS treatment (Fig. 3A to E), we also examined *virB* promoter activities in neutral AB-MES media and acidic or neutral 523 media with AS at 19°C for 40 h. The *virB* promoter activity was increased most (almost fivefold) in the *tzs* deletion mutant as compared with the wild type in acidic 523 media with AS induction (Fig. 4B). The increased degree of *virB* promoter activity in the *tzs* deletion mutant was the least for bacteria in neutral AB-MES media with AS (Fig. 4B). The *virB* promoter activities in the wild type and *tzs* deletion mutant were below the detection limits in AB-MES or 523 media without AS (data not shown). Additionally, the *virB* promoter activity in the *tzs* deletion mutant with AS induction was highest in acidic AB-MES media, followed by acidic 523 media, neutral 523 media, and neutral AB-MES media. These promoter activity assay results agree with protein accumulation levels determined by immunoblotting analyses (Fig. 3A and C to E), which suggests that the increase in VirB protein levels in the *tzs* deletion mutant may be regulated at the transcriptional level. Interestingly, in the wild type, *virB* promoter activities were induced by AS in neutral AB-MES media and acidic and neutral 523 media (Fig. 4B) but the levels of several AS-induced VirB proteins were detected only in

acidic 523 media (Fig. 3D), not in the other two media (Fig. 3C and E). The *virB* gene expression may be induced by AS in neutral AB-MES and 523 media but the VirB proteins might be unstable and could not be detected by immunoblotting analyses. Alternatively, *virB* promoter activity could be caused by its transcriptional fusion to the reporter gene expressed on the plasmid.

A. tumefaciens has adopted a VirA/VirG two-component sensory system to sense and react to AS and, subsequently, activate the transcription of *vir* genes and operons, including *virA*, *-B*, *-C*, *-D*, and *-E* (11,12,22,33). Therefore, we examined *virA*, *-B*, *-C*, *-D*, and *-E* and *tzs* promoter activities, with each promoter transcriptionally fused to the reporter gene *gfp* of the promoter probe plasmid pRU1156 and expressed in the wild type or *tzs* deletion mutant. The promoter activities of *virA*, *-B*, *-C*, *-D*, and *-E* and *tzs* were higher by 1.2- to 2.1-fold in the *tzs* deletion mutant than the wild type in acidic AB-MES media at 19°C with AS for 8 h (Fig. 4C and D; Supplementary Table 2). These data also suggested an increase of *virA* promoter activity in the *tzs* deletion mutant early (8 h) during AS induction which may, therefore, increase promoter activities of other *vir* genes and Vir protein level in the *tzs* deletion mutant. As well, promoter activity of *virE* was the highest in both the wild type and *tzs* deletion mutant, followed by *tzs*, *virD*, *virB*, and *virA*, with *virC* having the lowest promoter activity in both strains (Fig. 4C and D). These results were con-

Fig. 3. Absence of Tzs protein and exogenous cytokinin affect *Agrobacterium tumefaciens* virulence (Vir) protein accumulation during acetosyringone (AS) induction. Immunoblotting analysis of Vir protein levels in the wild-type (WT) and Δtzs mutant strains grown in acidic AB-MES media (pH 5.5) with 200 μ M AS at **A**, 19°C for 16 and 40 h or **B**, 25°C for 4 and 8 h. Molecular mass markers are indicated on the right (in kilodaltons) and Tzs, neomycin phosphotransferase II (NptII), and several Vir proteins are indicated on the left. Immunoblotting with NptII antibody was a loading control. Immunoblotting analysis of protein expression in WT and Δtzs mutant strains grown in **C**, neutral AB-MES media (pH 7) with AS at 19°C for 16 and 40 h; **D**, acidic 523 media (pH 5.5) with AS at 19°C for 16 and 40 h; **E**, neutral 523 media (pH 7) with AS at 19°C for 16 and 40 h; and **F**, acidic AB-MES media (pH 5.5) with AS at 19°C for 16 h with and without kinetin at 0.01 ppm.

sistent with previous observations of *virC* and *virE* showing the lowest and highest expression, respectively, for AS-inducible *vir* genes (51).

Supplementing exogenous cytokinin during AS induction repressed several Vir proteins and Tzs protein level in both the wild type and *tzs* deletion mutant (Fig. 3F). Therefore, we examined whether this repression is regulated at the transcriptional level. Both the wild-type and the *tzs* deletion mutant showed >25% reduction in *virA* promoter activity after the addition of kinetin at 0.01 ppm in the acidic AB-MES media with AS for 8 h at 19°C (Fig. 4C). Similarly, the *virB*, *-C*, *-D*, and *-E* and *tzs* promoter activities in both *A. tumefaciens* strains decreased 15 to 60% in the presence of exogenous kinetin during AS induction (Fig. 4C and D). *Arabidopsis* root segments were infected with the wild type or *tzs* deletion mutant on MS media at 22 to 24°C for 2 days in *Arabidopsis* stable or transient transformation assays; then, we examined *virB* promoter activity in both strains on MS media early (8 and 12 h) during AS induction. The promoter activity of *virB* was increased 7- and 26-fold in the *tzs* deletion mutant as compared with the wild type at 8 and 12 h, respectively, after the addition of AS on MS media (Fig. 4E; Supplementary Table 3). The *virB* promoter activities in the wild type decreased 60% after the addition of kinetin at 0.01 ppm at

both times on MS media with AS, whereas *virB* promoter activities in the *tzs* deletion mutant was decreased 70% under the same treatment (Fig. 4E). In summary, the *virA*, *-B*, *-C*, *-D*, and *-E* and *tzs* promoter activity results demonstrated that the increased VirB, VirD2, and VirE2 protein levels in the *tzs* deletion mutant may be caused by the increased *vir* promoter activities. In addition, the repression of *virA*, *-B*, *-C*, *-D*, and *-E* and *tzs* gene expression by exogenous cytokinin application during AS induction may be regulated at the transcriptional level.

The *tzs* deletion mutant showed impaired growth during AS induction and infection. Because the *tzs* deletion mutant showed increased AS-induced *vir* promoter activities and Vir protein accumulations during AS induction (Figs. 3 and 4), we examined other physiological changes with deletion of the *tzs* gene in *A. tumefaciens*. We first examined whether Tzs is critical for *A. tumefaciens* growth under AS-induced conditions when Vir proteins are expressed. The viable cell numbers of the wild-type and *tzs* deletion mutant were measured at 0, 16, 40, and 64 h after incubation in acidic AB-MES (minimal) media (pH 5.5) at 19°C with or without AS induction. The wild type and *tzs* deletion mutant did not differ in cell number without AS (Fig. 5A; Supplementary Table 4). As well, both the wild type and *tzs* deletion mutant grew more slowly with AS in acidic AB-MES

Fig. 4. *Agrobacterium tumefaciens* virulence (*vir*) promoter activities are affected by loss of function of Tzs protein and the addition of exogenous cytokinin. **A**, *virB* promoter *lacZ* activity analyses of the wild-type (WT), Δtzs mutant, and *tzs*-complemented ($\Delta tzs + tzs$) strains grown in acidic AB-MES media (pH 5.5) at 19°C for up to 40 h with acetosyringone (AS). **B**, *virB* promoter *lacZ* activity analysis of the WT and Δtzs mutant strains grown in neutral AB-MES media (pH 7), acidic (pH 5.5) or neutral (pH 7) 523 media at 19°C for 40 h with AS; * indicates $P < 0.05$ between the WT and Δtzs mutant strains by analysis of variance (ANOVA) or pairwise Student's *t* test. Promoter green fluorescent protein (GFP) analyses of **C**, *virA*, *virB*, *virC*, and *virD* and **D**, *virE* and *tzs* activity in the WT and Δtzs mutant in acidic AB-MES media (pH 5.5) at 19°C for 8 h with AS with and without kinetin at 0.01 ppm. **E**, *virB* promoter *lacZ* activity analyses of WT and Δtzs mutant on Murashige and Skoog (MS) medium (pH 5.7) at 22 to 24°C for 8 and 12 h with AS with and without kinetin at 0.01 ppm. The letter 'a' above each bar represents statistically significant differences between with and without exogenous cytokinin application for the same strain by ANOVA or pairwise Student's *t* test.

media at 19°C, and viable cell numbers were lower for the *tzs* deletion mutant than the wild type at the same time (Fig. 5A). In contrast, bacterial growth rates did not differ when both the wild type and the *tzs* deletion mutant were grown in the neutral AB-MES media (pH 7) at 19°C with and without AS (Fig. 5B). With acidic 523 media, the wild type and *tzs* deletion mutant grew more slowly with than without AS (Fig. 5C). In addition, in both acidic and neutral 523 media, the *tzs* deletion mutant grew more slowly than the wild type only with AS induction (Fig. 5C and D). Results of these experiments demonstrated that, when Vir protein levels were highly induced by AS in acidic AB-MES and 523 media (Fig. 3A and C), the *tzs* deletion mutant grew more slowly than the wild type or showed impaired growth under these growth conditions (Fig. 5A and C). In contrast, when Vir protein expression was not or barely induced by AS in acidic or neutral AB-MES or 523 media, the *tzs* deletion mutant showed growth rates similar to the wild type (Fig. 5A to D). These bacterial growth curve results also suggest that Tzs might be required for bacterial growth or cell viability in the acidic minimal and rich media at 19°C with AS induction.

The *vir* gene expression was increased in the *tzs* deletion mutant but virulence of the *tzs* deletion mutant was decreased in *Arabidopsis* (Fig. 2C to E). Therefore, we determined the bacterial growth rates of the *tzs* deletion mutant during *Agrobacterium* co-cultivation with *Arabidopsis* roots. We used an initial 100 µl of 10⁷ CFU ml⁻¹ bacterial concentrations to infect *Arabidopsis* roots on MS media at 22 to 24°C for 2 days and we determined the viable cell numbers of the wild type and the *tzs* deletion mutant at 0, 24, and 48 h during co-cultivation. Both *A. tumefaciens* strains grew faster at early times during co-cultivation periods with *Arabidopsis* roots (Fig. 5E; Supplementary Table 5). These results

were unlike the growth curves in acidic minimal AB-MES media with AS induction, in which viable cell numbers of both *A. tumefaciens* strains were reduced at 64 h after the addition of AS (Fig. 5A). In addition, viable cell numbers of the *tzs* deletion mutant were reduced to 17.6% that of the wild type at 24 h on co-cultivation with *Arabidopsis* roots, whereas viable cell numbers of the *tzs* deletion mutant were reduced to 35.1% that of the wild type at 24 h without co-cultivation with *Arabidopsis* roots (Fig. 5E and F). Thus, the *tzs* deletion mutant grew more slowly than the wild type early during infection of *Arabidopsis* roots on MS media (Fig. 5E). The growth rates of the *tzs*-complemented strain with (Fig. 5E) and without (Fig. 5F) co-cultivation with *Arabidopsis* roots were both faster than that of the *tzs* deletion mutant. The viable cell numbers of the *tzs*-complemented strain also recovered to almost the wild-type level on MS media with and without roots at 48 h (Fig. 5E, and F), which suggests that the absence of the *tzs* gene may cause growth defects of the *tzs* deletion mutant during both AS induction and infection.

Exogenous cytokinin affects *A. tumefaciens* growth rates during infection. The addition of exogenous cytokinin repressed AS-induced Vir protein levels in the *tzs* deletion mutant (Fig. 3F) but recovered the virulence of the *tzs* deletion mutant (Fig. 2C and 2E). Therefore, we examined whether supplementing exogenous cytokinin promoted bacterial growth and, therefore, enhanced bacterial infection ability. The cytokinin kinetin at 0.01 ppm was added during 48-h infection periods and viable cell numbers of the wild type and *tzs* deletion mutant were determined. The addition of exogenous cytokinin increased the viable cell numbers of both the wild type and *tzs* deletion mutant by 1.2- to 1.7-fold as compared with without cytokinin (Fig. 5E). In contrast, growth curves for the wild type and *tzs* deletion mutant did not differ

Fig. 5. Absence of Tzs protein and addition of exogenous cytokinin affect bacterial growth during infection. Bacterial growth curves of the wild-type (WT) and the Δtzs mutant with **A**, acidic AB-MES media (pH 5.5); **B**, neutral AB-MES media (pH 7); **C**, acidic 523 media (pH 5.5); or **D**, neutral 523 media (pH 7) at 19°C with or without acetosyringone (AS). Bacterial growth curves of the WT, Δtzs mutant, and *tzs*-complemented ($\Delta tzs + tzs$) strains with Murashige and Skoog (MS) media (pH 5.7) at 22 to 24°C **E**, with or **F**, without roots in the absence or presence of kinetin at 0.01 ppm. The y-axis in the figure was labeled as the log scale.

after adding cytokinin in MS media without *Arabidopsis* roots (Fig. 5F). The presence of exogenous cytokinin may enhance bacterial growth only during infection of roots when *vir* genes were expressed.

DISCUSSION

Plants are sessile and continuously communicate with the pathogenic and nonpathogenic microbes in the rhizosphere and phylloplane during plant growth and development. A wide range of plant-associated bacteria and fungi, including *Agrobacterium*, *Pseudomonas*, *Rhizobium*, *Rhodococcus*, *Erwinia*, *Plasmodiophora*, *Puccinia*, and *Azospirillum* spp., produce phytohormones such as auxins and cytokinins to affect plant cell divisions, leading to tumors or gall formations, or to stimulate root growth and modify root architectures, leading to increased nutrient and water absorption (14,15,21,35,37,59). During *A. tumefaciens* infection, the wild-type T-DNA transfers from the bacterium into plant cells and integrates into plant genomes, thus resulting in the production of auxins and cytokinins and tumor formation in plants. Unlike the T-DNA-encoded cytokinin biosynthesis gene, the *tzs* gene in the nopaline *A. tumefaciens* strain is not translocated into plant cells during infection, and its gene product causes the synthesis of *trans*-zeatin in bacterial cells on induction of virulence genes (20,28,38). In this study, we successfully established the involvement of Tzs or its cytokinin in the *A. tumefaciens* infection process, including regulation of *vir* gene expression and bacterial growth, by genetic study of the *A. tumefaciens* *tzs* deletion mutant. Both genetic and chemical complementation assays demonstrated that the *tzs* gene inserted in the chromosome locus and exogenous application of the cytokinin kinetin at 0.01 ppm (0.05 μ M) complemented the reduced virulence phenotype of the *tzs* deletion mutant. Furthermore, absence of the *tzs* gene in the *A. tumefaciens* and supplementing cytokinin at 0.01 ppm affected the accumulation of several AS-induced Vir proteins, *vir* gene promoter activities, and bacterial growth during AS treatments.

Tzs protein expression was upregulated by AS and even further by the acidic environment of the culture media. The above data are consistent with a previous study showing that zeatin secretion from wild-type *A. tumefaciens* C58 was maximal at pH 5.0 and significantly decreased at pH 7.0 in the presence of 200 μ M AS (38). Interestingly, zeatin production was also maximal at pH 5.0 in noninduced *A. tumefaciens* C58 cultures, and the level was almost 100-fold lower than that in induced cultures (38). However, we did not observe a significant increase in Tzs protein expression by media with acidic pH without AS (Fig. 1). Similar to our results, the *tzs* gene is not induced by acid alone in recent microarray analyses of *A. tumefaciens* C58 (64). In contrast to other known virulence proteins, Tzs protein is induced by AS even in minimal or rich media at neutral pH. Examining whether Tzs protein expression is still regulated by VirA and VirG or other regulatory proteins in *A. tumefaciens* at neutral pH is of interest.

Higher levels of Vir protein with AS induction in the *tzs* deletion mutant than the wild type were most obvious for VirD2 and VirB2; followed by VirB9, -10, and -11; and then VirB4, VirB8, and VirE2 (Fig. 3A and B). The different AS-induced Vir protein levels may relate to Vir protein functions and stability in *A. tumefaciens* during infection. VirD2 protein, one of the most important and versatile AS-induced Vir proteins, participates in T-DNA processing, transfer, and integration during *A. tumefaciens* infection. The major component of T-pilus, VirB2 protein, mediates the T-DNA and Vir protein transfer from bacteria into plant cells. Both VirD2 and VirB2 proteins play important and essential roles during *A. tumefaciens* infection; therefore, these Vir protein levels in *A. tumefaciens* may significantly affect bacterial virulence. Interestingly, the protein level of VirB8, a structural component of the type IV secretion system, was less

affected by the absence of the *tzs* gene, which might be due to the presence of the small heat-shock protein HspL as the VirB8 chaperone (56). The increased amount of AS-induced Vir proteins in the *tzs* deletion mutant compared with the wild type was evident not only in acidic minimal medium but also when bacteria grown in rich media with neutral or acidic pH and in minimal media with neutral pH (Fig. 3C to E). Furthermore, no AS-induced Vir proteins were detected in the wild type in rich or minimal media with neutral pH (Fig. 3C and E), which is consistent with previous results (12,25,44,67).

Previous studies have demonstrated greater AS-induced *vir* gene level in acidic minimal media than acidic rich media (23, 41,61). In this study, we also observed higher *virB* promoter activity in the wild-type *A. tumefaciens* in acidic minimal than acidic rich media ($2,815.9 \pm 17.1$ versus 86.0 ± 15.2 Miller units) (Fig. 4A and B) at 40 h after AS treatment at 19°C. In addition to examining *virB* promoter activity, we examined *virA*, -C, -D, and -E and *tzs* promoter activities in the wild type and *tzs* deletion mutant with and without exogenous cytokinin during AS induction. Promoter activities of several AS-induced *vir* genes were increased in the *tzs* deletion gene in *A. tumefaciens* and decreased with exogenous cytokinin. Importantly, *virA* promoter activity increased 1.2-fold in the *tzs* deletion mutant and decreased almost 25% in both the wild type and *tzs* deletion mutant after the addition of cytokinin. Tzs or exogenous cytokinin may play a negative role in regulating *vir* and *tzs* gene expression at the transcriptional level and possibly via the VirA/VirG two-component system with AS induction. One of the *A. tumefaciens* T-DNA-encoded products, IAA, inhibits virulence gene induction in vitro by competing with AS (32). Another phytohormone, SA, also inhibit bacterial growth and virulence gene induction and, therefore, affects *A. tumefaciens* virulence (6). In addition to phytohormones, other environmental factors, including sugar, temperature, and acidity, influence *vir* gene expressions directly or indirectly through the VirA and VirG proteins. A chromosomally encoded two-component system, ChvG/ChvI, helped *A. tumefaciens* detect extracellular acidity and activate downstream *virG* expression (11,12). The combination of several environmental factors and genetic factors controls *vir* gene expressions in *A. tumefaciens* and displays the complex network for *vir* gene regulations. Further examining whether cytokinin could directly or indirectly regulate *vir* gene expression through the VirA/VirG system during infection would be of interest. One possible mechanism might be competition with AS for the direct binding of the VirA protein or indirectly affecting *vir* gene expression by other unknown two-component systems that respond to cytokinin.

In this study, we found the Vir protein levels negatively related to growth rates of the *tzs* deletion *A. tumefaciens* mutant with AS induction in acidic AB-MES minimal and 523 rich media. Vir protein expression is required for *A. tumefaciens* T-DNA transfer and tumorigenesis in plants. However, the increased Vir protein level in the *tzs* deletion mutant could not significantly enhance its virulence in infecting *Arabidopsis*, which may be due to the lower bacterial growth rates of the *tzs* deletion mutant during infection. Abnormally high levels of Vir proteins, including VirD2 and several VirB proteins, in the *tzs* deletion mutant might affect other normal physiological metabolic functions of bacteria and influence bacterial growth rates during *A. tumefaciens* infection. However, our tumor assay results of cowpea and green pepper showed that the *tzs* deletion mutant increased its virulence on these two plant species. The reasons underlying the higher virulence of *tzs* mutants on these two plant species remain unclear. One possible hypothesis might be that the wild-type strain might outgrow the *tzs* mutant under infections: 100 μ l of 10^9 CFU ml⁻¹ bacteria cultures of the *tzs* mutant might be an adequate amount to successfully infect cowpea and green pepper whereas the wild-type strain, with excessive bacterial quantity, may adversely affect transformation efficiencies. Further examination of bacterial growth

rates and vir protein accumulations during infections of cowpea and green pepper might help us understand more about the Tzs functions in these two plant species.

In conclusion, the *tzs* deletion mutant conferred higher *vir* gene expression during AS induction but slower bacterial growth rates and virulence during infection than the wild-type *A. tumefaciens*. The increased AS-induced Vir protein accumulation in the *tzs* deletion mutant may affect its bacterial growth rate and decrease its virulence in several plant species for a role of *tzs* in *A. tumefaciens* infection. Toward the end of *A. tumefaciens* infection, auxin could inhibit both agrobacterial growth and *vir* gene expression with the indication of successful *A. tumefaciens*-mediated plant transformation. Cytokinin, unlike auxin, inhibited *vir* gene expression at the early stages of *A. tumefaciens* infection but promoted agrobacterial growth when co-incubated with *Arabidopsis* roots. This study demonstrated the early involvement of the Tzs protein during *A. tumefaciens* infection and that cytokinin secretion by induced *A. tumefaciens* may create nutrient sinks at the infected sites and facilitate agrobacterial growth and colonization.

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