Agrobacterium-produced and exogenous cytokinin-modulated Agrobacterium-mediated plant transformation

HAU-HSUAN HWANG^{1,2*}, MING-HSUAN WANG¹, YING-LING LEE², YUN-LONG TSAI¹, YI-HO LI², FONG-JHIH YANG², YU-CHEN LIAO¹, SHAO-KAI LIN¹ AND ERH-MIN LAI^{1,*}

¹Institute of Plant and Microbial Biology, Academia Sinica, Taipei 115, Taiwan

²Department of Life Sciences, National Chung-Hsing University, Taichung 402, Taiwan

SUMMARY

Agrobacterium tumefaciens is a plant pathogenic bacterium that causes neoplastic growths, called 'crown gall', via the transfer and integration of transferred DNA (T-DNA) from the bacterium into the plant genome. We characterized an acetosyringone (AS)-induced tumour-inducing (Ti) plasmid gene, *tzs* (*trans*-zeatin synthesizing), that is responsible for the synthesis of the plant hormone cytokinin in nopaline-type *A. tumefaciens* strains. The loss of Tzs protein expression and *trans*-zeatin secretions by the *tzs* frameshift (*tzs-fs*) mutant is associated with reduced tumorigenesis efficiency on white radish stems and reduced transformation efficiencies on *Arabidopsis* roots. Complementation of the *tzs-fs* mutant with a wild-type *tzs* gene restored wild-type levels of *trans*-zeatin secretions and transformation efficiencies. Exogenous application of cytokinin during infection increased the transient transformation efficiency of *Arabidopsis* roots infected by strains lacking Tzs, which suggests that the lower transformation efficiency resulted from the lack of *Agrobacterium*-produced cytokinin. Interestingly, although the *tzs-fs* mutant displayed reduced tumorigenesis efficiency on several tested plants, the loss of Tzs enhanced tumorigenesis efficiencies on green pepper and cowpea. These data strongly suggest that Tzs, by synthesizing *trans*-zeatin at early stage(s) of the infection process, modulates plant transformation efficiency by *A. tumefaciens*.

INTRODUCTION

Agrobacterium tumefaciens genetically transforms the host cells of a wide range of plant species (DeCleene and DeLey, 1976). During infection, a defined piece of the tumour-inducing (Ti) plasmid, the transferred DNA (T-DNA), is transported from the bacterium into the nucleus of plant cells, where it becomes

integrated into the plant genome (Citovsky *et al.*, 2007; Gelvin, 2003; McCullen and Binns, 2006). In nature, wounded plants secrete a sap with a characteristic acidic pH (5.0–5.8) and high content of different phenolic compounds, some of which are recognized by *A. tumefaciens* and induce virulence (*vir*) gene expression. The best-studied and most effective *vir* gene inducers are monocyclic phenolics, such as acetosyringone (AS; Stachel *et al.*, 1985). Following *vir* gene induction, the T-DNA is processed from the Ti plasmid and, together with several effector proteins, is transferred from the bacterium into the host plant cells via a VirB/D4-encoded type IV secretion system (T4SS) (Christie *et al.*, 2005). In plants, the expression of several oncogenes encoded by T-DNA directs the production of the plant growth hormones auxin and cytokinin that are responsible for the proliferation of transformed plant cells (Akiyoshi *et al.*, 1984; Montoya *et al.*, 1977).

The products of the T-DNA genes *indoleacetamide hydrolase* (*iaaH*) and *tryptophan monooxygenase* (*iaaM*), and *isopentenyl transferase* (*ipt*) (*tmr*), catalyse the production of auxin and cytokinin, respectively. The T-DNA *ipt* gene encodes isopentenyl transferase (IPT) which converts dimethylallyl diphosphate (DMAPP) and adenosine monophosphate (AMP) to form the cytokinin isopentenyladenosine 5'-monophosphate (iPMP) (Akiyoshi *et al.*, 1983, 1984; Barry *et al.*, 1984; Sakakibara, 2005, 2006). The *trans*-zeatin synthesizing (*tzs*) gene, localized in the *vir* region of nopaline-type Ti plasmids, encodes a cytokinin biosynthetic prenyl transferase, which shares considerable homology with IPT encoded by T-DNA (Akiyoshi *et al.*, 1985, 1987; Beaty *et al.*, 1986; Powell *et al.*, 1988). In addition to utilizing DMAPP and AMP to produce cytokinin iPMP, Tzs protein can metabolize 4-hydroxy-3-methyl-2-(*E*)-butenyl diphosphate (HMBDP) and directly produce zeatin riboside 5'-phosphate (ZMP) (Krall *et al.*, 2002). Plant-encoded IPT predominantly utilizes DMAPP synthesized from the major isoprenoid pathway, the mevalonic acid (MVA) pathway; however, both the *vir* regulon-encoded Tzs and the T-DNA-encoded IPT proteins probably use HMBDP as a substrate to synthesize *trans*-zeatin (Krall *et al.*, 2002; Sakakibara, 2005, 2006; Sugawara *et al.*, 2008). HMBDP is

*Correspondence: Email: emlai@gate.sinica.edu.tw; hauhsuan@dragon.nchu.edu.tw

a metabolic intermediate synthesized from the alternative methylerythritol phosphate (MEP) pathway, which occurs in bacteria and plastids of higher plants (Sakakibara, 2005, 2006). The recent resolved crystal structure of Tzs, in combination with site-directed mutagenesis studies, identified the amino acid residues Asp-173 and His-214 as being responsible for the substrate specificity differences between plant (*Arabidopsis*) and bacterial (*Agrobacterium*) IPTs (Sugawara *et al.*, 2008). A previous study has demonstrated that auxin and cytokinin pretreatment of petiole explants from transformation-recalcitrant *Arabidopsis* ecotypes significantly enhances their susceptibility to transformation (Chateau *et al.*, 2000). This study demonstrated that exogenous auxin and cytokinin treatments before *Agrobacterium* infections could have positive effects on the subsequent genetic transformations.

Unlike the *ipt* gene, the *vir* regulon-encoded *tzs* gene is not incorporated into the host plant genome during infection. The *tzs* gene is constitutively expressed at low levels and is induced by AS in *A. tumefaciens* (Anand *et al.*, 2008; Cho and Winans, 2005; John and Amasino, 1988; Lai *et al.*, 2006; Powell *et al.*, 1988; Yuan *et al.*, 2007). In addition, *virA* and *virG* are necessary for phenolic-induced Tzs protein expression and *trans*-zeatin production (John and Amasino, 1988; Lai *et al.*, 2006; Powell *et al.*, 1988). The *tzs* gene may promote transformation, because root induction efficiency was higher when an *Agrobacterium rhizogenes* strain lacking the *tzs* gene was co-inoculated on flax (*Linum usitatissimum* L.) cotyledon explants with a disarmed *A. tumefaciens* strain containing the *tzs* gene (Zhan *et al.*, 1990). Interestingly, Tzs protein was detected in the membrane fraction and on the cell surface of *A. tumefaciens* (Aly *et al.*, 2008; Lai *et al.*, 2006) and interacted with VirB5, a component of T4SS and T-pilus (Schmidt-Eisenlohr *et al.*, 1999), in yeast and *in vitro* (Aly *et al.*, 2008). However, whether or how Tzs contributes to the virulence of *A. tumefaciens* remains unclear. In this study, we investigated the importance and possible role(s) of Tzs protein and cytokinin in

Agrobacterium virulence. The data together suggest that Tzs and its catalysed product cytokinin modulate the transformation efficiency at early step(s) of *Agrobacterium* infection.

RESULTS

Tzs protein is responsible for *trans*-zeatin secretion and promotes virulence on white radish

In order to investigate whether the *tzs* gene is involved in virulence, we generated a *tzs* mutant in the C58-derived virulent strain NT1RE(pJK270). This *tzs* mutant, named *tzs-fs*, contains a single base pair deletion in the *tzs* gene to create a frameshift mutation predicted to result in a 25-kDa truncated protein retaining a short Tzs N-terminus (17 amino acids). Although 27-kDa wild-type Tzs protein was highly expressed when the wild-type strain NT1RE(pJK270) was grown in AB-MES medium with AS at 19 °C for 40 h, no Tzs protein expression was detected in the *tzs-fs* mutant from immunoblot analysis (Fig. 1A). Under the same induction conditions, NT1RE(pJK270) secreted 492.2 µg/L *trans*-zeatin into the culture medium, as assayed by ultra-performance liquid chromatography (UPLC) of AS-induced culture medium (Fig. 1B). As expected, the loss of Tzs protein was correlated with the deficiency of *trans*-zeatin secretion in the *tzs-fs* mutant (Fig. 1B). To determine whether Tzs protein contributes to virulence, we performed tumour assays on stems of white radish plants. The *tzs-fs* mutant remained virulent, but fewer plants formed tumours than with the wild-type *A. tumefaciens* strain NT1RE(pJK270) (Fig. 1C; Table S1, see Supporting Information). We also included a Ti-plasmidless *A. tumefaciens* strain, NT1RE, as a negative control, which did not cause tumour formations on white radish (data not shown).

To confirm that the loss of *trans*-zeatin secretion and reduced virulence resulted from the loss of Tzs protein in the *tzs-fs* mutant, we performed a genetic complementation test. We introduced the

Fig. 1 Tzs is responsible for *trans*-zeatin secretion and promotes *Agrobacterium tumefaciens* virulence on white radish stems. (A) Immunoblot analyses of Tzs and neomycin phosphotransferase II (NptII) in the wild-type (WT) (NT1RE[pJK270]), *tzs-fs* mutant (*tzs-fs*) and *tzs*-complemented (*tzs-fs* + *tzs*) strains grown in AB-MES in the presence of 200 µM acetosyringone (AS) at 19 °C for 40 h. Equal amounts of protein were resolved by Tricine-sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and analysed by immunoblotting with antibodies against Tzs and NptII, which served as a loading control. The molecular weight markers are indicated on the left in kilodaltons. (B) Concentration of *trans*-zeatin in the culture medium of the wild-type, *tzs-fs* mutant and *tzs*-complemented strains by ultra-performance liquid chromatography (UPLC). Culture medium from each bacterial strain grown in AB-MES containing AS was subjected to UPLC analysis, and *trans*-zeatin was eluted by methanol at a specific retention time (min, x-axis) and detected by absorbance at 265 nm wavelength (y-axis). The mean concentration of *trans*-zeatin (µg/L) detected in each sample from three independent experiments is shown with standard deviations. N.D., not detected. The lowest concentration of *trans*-zeatin detected with our UPLC system was 100 µg/L, whereas no signal could be detected when 10 µg/L of *trans*-zeatin was analysed (data not shown). (C) Virulence of the wild-type, *tzs-fs* mutant and *tzs*-complemented strains on white radish stems. The tumorigenesis efficiency of white radish stems infected with the wild-type strain NT1RE(pJK270), with approximately 80%–100% of plants forming tumours in each experiment, was set at 100%, and that of the mutant is shown relative to that of the wild-type strain. Mean values for relative tumorigenesis frequencies from at least three independent experiments are shown with standard errors. An asterisk above each mean represents a statistically significant difference between wild-type (NT1RE[pJK270]) and *tzs-fs* mutant strains based on analysis of variance (ANOVA) and pairwise Student's *t*-test. *P* < 0.05 for all significant differences between strain combinations.

A

B

C

Fig. 2 The *tzs-fs* mutant shows lower tumorigenesis and transient transformation efficiency in *Arabidopsis* roots. Root segments from 3–4-week-old *Arabidopsis* plants were infected with the wild-type NT1RE(pJK270), *tzs-fs* mutant and *tzs*-complemented *Agrobacterium tumefaciens* strains at two concentrations [10^8 and 10^7 colony-forming units (cfu)/mL] for both tumorigenesis (A) and transient transformation (B) assays. The transformation efficiency of root segments infected with the wild-type strain NT1RE(pJK270), with approximately 40%–70% of roots forming tumours or β -glucuronidase (GUS) activity in each experiment, was set at 100% and that of the mutant is shown relative to that of the wild-type strain. Mean values of virulence from at least three independent experiments are shown with standard errors. (C) Representative plates of *Arabidopsis* roots with tumours infected at a bacterial concentration of 10^8 cfu/mL.

plasmid pIncP-Tzs, with the *tzs* gene expressed under the control of its native promoter, into the wild-type and *tzs-fs* mutant strains. The expression of the wild-type *tzs* gene in the mutant strain restored Tzs protein expression and *trans*-zeatin secretion to levels slightly higher than those of the wild-type (Fig. 1A,B), probably because of the increased copy number of the *tzs* gene when expressed in plasmid. Neither Tzs protein nor secreted *trans*-zeatin could be detected in the *tzs-fs* mutant alone or with the empty vector (Fig. 1A,B). The *tzs*-complemented strain caused almost 100% of white radish plants to develop tumours (Fig. 1C; Table S1), which indicates that the reduced virulence phenotype of the *tzs-fs* mutant resulted from the loss of a functional Tzs protein. These data show that Tzs protein and its synthesized product *trans*-zeatin are not essential, but may play an important role in *A. tumefaciens* virulence.

Tzs promotes virulence on *Arabidopsis* roots at step(s) prior to T-DNA integration

Because tumorigenesis is caused by the overproduction of auxin and cytokinin produced in T-DNA-transformed cells, we next investigated whether Tzs promotes *Agrobacterium* virulence at step(s) prior to T-DNA integration or during the process of tumorigenesis after T-DNA integration.

We performed quantitative tumorigenesis and transient transformation assays using the T-DNA binary vector pCAMBIA2201-Gm harbouring a *gusA*-intron reporter gene [the intron was utilized to prevent the expression of β -glucuronidase (GUS) activity in *A. tumefaciens*] (Nam *et al.*, 1997, 1998, 1999; Zhu *et al.*, 2003). We determined the tumorigenesis and transient transformation efficiency based on the percentage of *Arabidopsis* roots that had formed tumours 1 month after infection and that showed GUS activity 7 days after infection, respectively. In both assays, the *tzs-fs* mutant was less than 50% as virulent as the wild-type strain (Fig. 2A–C; Tables S2 and S3, see Supporting information). The *tzs*-complemented strain restored the reduced tumorigenesis efficiency of the *tzs-fs* mutant on *Arabidopsis* roots to 90% and 60% of the wild-type level when inoculated at 10^8 and 10^7 colony-forming units (cfu)/mL, respectively (Fig. 2A; Table S2). The representative results of *Arabidopsis* roots with tumorigenesis assays when infected with a bacterial concentration of 10^8 cfu/mL are shown in Fig. 2C. In transient transformation assays, the *tzs*-complemented strain restored the reduced virulence of the *tzs-fs* mutant to 80%–90% of the wild-type level when inoculated at either 10^8 or 10^7 cfu/mL (Fig. 2B; Table S3). Because transient transformation does not require the integra-

Fig. 3 Cytokinin supplementation during infection increases the transient transformation efficiencies of strains lacking Tzs. *Arabidopsis* root segments were infected with a bacterial concentration of 10^7 colony-forming units (cfu)/mL of various *Agrobacterium tumefaciens* strains, the wild-type (NT1RE[pJK270]), the *tzs-fs* mutant, A208, LBA4404 and EHA101, on Murashige and Skoog (MS) medium with or without different kinetin concentrations for 2 days. Seven days after infection, root segments were stained with 5-bromo-4-chloro-3-indolyl- β -D-glucuronic acid (X-gluc) staining solution. Mean values of the transformation frequencies from at least three independent experiments are shown with standard errors. An asterisk above each mean represents a statistically significant difference between the transformation frequencies of the wild-type and various tested strains in the absence of exogenous cytokinins during infection based on analysis of variance (ANOVA) and pairwise Student's *t*-test. $P < 0.05$ for all significant differences between strains. The letters 'a', 'b' and 'c' above each mean represent statistically significant differences with and without exogenous cytokinins using the same strain during infection based on ANOVA and pairwise Student's *t*-test. GUS, β -glucuronidase.

tion of T-DNA into the plant genome (Mysore *et al.*, 1998), these data suggest the involvement of Tzs at the early step(s) of the transformation process prior to T-DNA integration.

The addition of cytokinin during infection enhances the transient transformation efficiency on *Arabidopsis* roots in strains lacking Tzs

To examine further whether the reduced virulence of the *tzs-fs* mutant results from the lack of cytokinin production by *A. tumefaciens*, we determined whether the addition of cytokinin during infection could enhance virulence. *Arabidopsis* roots infected with 10^7 cfu/mL of bacteria were co-cultivated on Murashige and Skoog (MS) medium containing various concentrations of a cytokinin, kinetin (0, 0.01, 0.3, 0.5, 1, 2.5, 5, 8, 10 and 15 mg/L). The transient transformation efficiencies of the *tzs-fs* mutant with treatments of kinetin from 0.01 to 15 mg/L were higher than those without kinetin treatment (Fig. 3). In contrast, the transient transformation efficiency was not significantly increased when kinetin was added during co-cultivation with the respective wild-type strain NT1RE(pJK270) (Fig. 3). The results of the combination of genetic and chemical complementation experi-

ments strongly suggest that the reduced virulence phenotype of the *tzs-fs* mutant is caused by the absence of cytokinin secretion by *A. tumefaciens* during the early stage of infection.

We further examined whether cytokinin supplementation during co-cultivation could enhance the virulence of *A. tumefaciens* LBA4404 and EHA101, two strains lacking the *tzs* gene. As shown in Fig. 3, the transient transformation efficiencies were below 5% for LBA4404 and EHA101 when no cytokinins were added during infection. With kinetin treatment of 0.01–15 mg/L during infection, the transformation efficiencies of LBA4404 and EHA101 increased two- to five fold (Fig. 3). These data suggest that the exogenous addition of cytokinin during infection can enhance the transformation efficiencies of *Arabidopsis* roots infected by *A. tumefaciens* strains deficient in cytokinin secretion.

The addition of cytokinin enhances the transient transformation efficiencies on *Arabidopsis* roots infected with NT1RE(pJK270) at lower bacterial concentrations

The transient transformation efficiencies of *Arabidopsis* roots infected by the wild-type nopaline strain NT1RE(pJK270) were

Fig. 4 Addition of cytokinin enhances transient transformation efficiencies on *Arabidopsis* roots infected with NT1RE(pJK270) at lower bacterial concentrations. *Arabidopsis* root segments were infected with different concentrations [10^5 , 10^6 , 10^7 and 10^8 colony-forming units (cfu)/mL] of the wild-type *Agrobacterium tumefaciens* strain NT1RE(pJK270) on Murashige and Skoog (MS) medium with or without kinetin for 2 days. Root segments were stained with 5-bromo-4-chloro-3-indolyl- β -D-glucuronic acid (X-gluc) staining solution 7 days after infection. The mean values of the transformation frequencies from at least three independent experiments are shown with standard errors. Letters 'a' or 'b' above each mean represent statistically significant differences between the presence and absence of exogenous cytokinins with the same wild-type bacterial concentration during infection based on analysis of variance (ANOVA) and pairwise Student's *t*-test. GUS, β -glucuronidase.

not increased significantly with kinetin treatments with a bacterial concentration of 10^7 cfu/mL used in these assays (Fig. 3). Similar results were observed with another nopaline-type *A. tumefaciens* strain, A208 (Fig. 3). It is possible that sufficient amounts of cytokinin were secreted by the wild-type nopaline *A. tumefaciens* strains when roots were infected at 10^7 cfu/mL. We therefore performed transient transformation assays with different concentrations, from 10^5 to 10^8 cfu/mL, of the wild-type nopaline *A. tumefaciens* strain NT1RE(pJK270). Figure 4 shows no significant increase in transient transformation efficiency in cytokinin-treated roots infected with 10^7 – 10^8 cfu/mL bacteria. However, when lower bacterial concentrations (10^6 and 10^5 cfu/mL) were used, the transient transformation efficiencies increased at least two fold when 8 and 10 mg/L of cytokinin were added to the medium during infection (Fig. 4). Thus, the addition of cytokinin when *Arabidopsis* roots were infected by lower bacterial concentrations of *A. tumefaciens*, which presumably secrete lower levels of *trans*-zeatin, may provide adequate amounts of cytokinin to assist *Agrobacterium*-mediated transformation efficiency.

Tzs affects virulence to different extents on different plant species

The Ti plasmid determines, in part, the host range of *A. tumefaciens* (Kao *et al.*, 1982; Loper and Kado, 1979). Octopine and nopaline strains of *A. tumefaciens* differ in their ability to induce tumours on *Nicotiana glauca* (Jarchow *et al.*, 1991; Melchers *et al.*, 1990; Otten *et al.*, 1985). To determine whether Tzs affects virulence on various plant species, we performed tumour assays on 30 plant species (Table 1). The *tzs-fs* mutant reduced virulence by approximately 20%–40% on potato (*Solanum tuberosum*) tuber discs, stems of pai-tsai (*Brassica rapa* L. R. Chinensis Group), loose leaf lettuce (*Lactuca sativa* L. var. *crispa*) and carnation (*Dianthus caryophyllus* L.) when compared with the wild-type strain NT1RE(pJK270) (Fig. 5A,B; Tables S4 and S5, see Supporting Information). In contrast, the *tzs-fs* mutant resulted in higher virulence on the stems of cowpea (*Vigna unguiculata* cv. Green Pod Kaoshiung) and green pepper (*Capsicum annuum* cv. Vega) (Fig. 5C; Table S5). No significant differences in virulence between the wild-type *A. tumefaciens* and the *tzs-fs*

Table 1 Summary of tumour assay results on various plant species.

Effects of <i>tzs-fs</i> mutation on virulence	Family name	Test host
Reduced transformation efficiency	Brassicaceae	<i>Arabidopsis thaliana</i> White radish (<i>Raphanus sativus</i> cv. Rosy) Pai-tsai (<i>Brassica rapa</i> L. R. Chinensis Group)
	Asteraceae	Loose leaf lettuce (<i>Lactuca sativa</i> L. var. <i>crispa</i>)
	Solanaceae	Potato (<i>Solanum tuberosum</i>)
	Caryophyllaceae	Carnation (<i>Dianthus caryophyllus</i> L.)
Enhanced transformation efficiency	Solanaceae	Green pepper (<i>Capsicum annuum</i> cv. Vega)
No difference	Leguminosae	Cowpea (<i>Vigna unguiculata</i> cv. Green Pod Kaoshiung)
	Brassicaceae	Ching chiang pai-tsai (<i>Brassica chinensis</i>) Broccoli (<i>Brassica oleracea</i> L. var. <i>Italica</i> Group) Kale (<i>Brassica oleracea</i>) Head mustard (<i>Brassica juncea</i> var. <i>capitata</i> Hort.) Chinese cabbage (<i>Brassica campestris</i> L. <i>Pekinensis</i> Group) Matthiola (<i>Matthiola incana</i> L. R. Br.)
		Sunflower (<i>Helianthus annuus</i>) Gazania (<i>Gazania splendens</i>)
		Pointed leaf lettuce (<i>Lactuca indica</i> L. var. <i>idivisa</i> (Mak.) Hara) Romaine lettuce (<i>Lactuca sativa</i> L. var. <i>romana</i>) Chicory (<i>Cichorium intybus</i> L.)
		Tomato (<i>Lycopersicon esculentum</i> cv. microtone) Tobacco (<i>Nicotiana tobacum</i> cv. W38) Egg plant (<i>Solanum melongena</i> L. var. <i>esculentum</i>) Petunia (<i>Petunia hybrida hort.</i>)
		Carrot (<i>Daucus carota</i> cv. Red Sky) Cilantro (<i>Coriandrum sativum</i>) Celery (<i>Apium graveolens</i> L.)
		Kidney bean (<i>Phaseolus vulgaris</i>) Amaranth (<i>Amaranthus mangostanus</i> L.) Spinach (<i>Spinacia oleracea</i>)
	Cucurbitaceae	Cucumber (<i>Cucumis sativus</i> cv. Merry Swallow)
	Apocynaceae	Vinca (<i>Catharanthus roseus</i>)

mutant were observed with other plant species tested (Tables 1 and S5). These data suggest that Tzs and/or its cytokinin product may play different roles when *A. tumefaciens* infects different host plants.

DISCUSSION

Many pathogenic fungi and bacteria that are intimately associated with plants, including *Pseudomonas*, *Agrobacterium*, *Rhizobium* and *Azospirillum* species, can produce and secrete substantial amounts of cytokinins and/or cause plant cells to synthesize plant hormones (Costacurta and Vanderleyden, 1995; Goethals *et al.*, 2001; Morris, 1986; Panopoulos and Peet, 1985; Walters and McRoberts, 2006). These plant hormones contribute to pathogenesis by some microorganisms and, in other cases, may be involved in root growth stimulation by beneficial bacteria and associative symbiosis (Costacurta and Vanderleyden, 1995; Walters and McRoberts, 2006). In this study, we characterized the effect of the *tzs* gene on *A. tumefaciens* virulence. The reduced transformation efficiency caused by the *tzs-fs* mutant compared with its wild-type counterpart NT1RE(pJK270) on

several plant species tested (Figs 1C, 2, 3, and 5) indicates a role for Tzs in *Agrobacterium* virulence.

The *vir* regulon-encoded *tzs* gene is constitutively expressed at low levels and is induced by AS in *A. tumefaciens* (Anand *et al.*, 2008; Cho and Winans, 2005; John and Amasino, 1988; Lai *et al.*, 2006; Powell *et al.*, 1988; Yuan *et al.*, 2007). AS-induced Tzs protein expression and *trans*-zeatin secretion (Fig. 1A,B; Lai *et al.*, 2006; Powell *et al.*, 1988) suggest a possible role for Tzs in the promotion of *Agrobacterium* virulence. From our finding that the *tzs-fs* mutant caused the reduction of both tumorigenesis and transient transformation on *Arabidopsis* roots (Fig. 2A,B), we suggest that Tzs is involved in the early step(s) prior to T-DNA integration. Because Tzs functions as an IPT for *trans*-zeatin biosynthesis, the role of Tzs in *Agrobacterium* virulence could be contributed by *trans*-zeatin. This hypothesis was supported by results of our chemical complementation assays with the cytokinin kinetin. Exogenous application of kinetin increased the ability of the *tzs-fs* mutant bacteria to transiently transform *Arabidopsis* roots (Fig. 3). In addition, the presence of exogenous cytokinin during infection enhanced the transient transformation efficiencies of two *tzs*-lacking strains, LBA4404 and EHA101

Fig. 5 The *tzs-fs* mutant decreased virulence on potato tubers, pai-tsai, loose leaf lettuce and carnation and caused more plants to produce tumours on cowpea and green pepper. Potato tuber discs (A), stems of pai-tsai, loose leaf lettuce and carnation (B), stems of cowpea and green pepper (C) were infected with the wild-type NT1RE(pJK270) and *tzs-fs* mutant as indicated. Tumours were scored 5 weeks after infection. The tumorigenesis efficiency of potato tuber discs (A) infected with the wild type was approximately 20–30 tumours per disc, and those of pai-tsai, loose leaf lettuce and carnation (B) were approximately 40%–90% of stems forming tumours in each experiment. The tumorigenesis efficiency of the wild-type shown in (A) and (B) was set at 100% and that of the mutant is shown relative to that of the wild-type strain. The transformation efficiency of stems of cowpea and green pepper (C) infected with the wild-type *Agrobacterium tumefaciens* strain NT1RE(pJK270), with approximately 10%–30% of stems forming tumours in each experiment, was set at 10% and that of the mutant is shown relative to that of the wild-type strain. Mean values of the tumorigenesis frequencies from at least three independent experiments are shown with standard errors. An asterisk above each mean represents a statistically significant difference between the wild-type and various tested strains based on analysis of variance (ANOVA) and pairwise Student's *t*-test. $P < 0.05$ for all significant differences between strains.

(Fig. 3). The levels of increased transient transformation efficiencies in the *tzs-fs* mutant treated with kinetin were independent of the kinetin concentrations used in our experiments. These results could indicate that the lowest kinetin concentration, 0.01 mg/L, used in the chemical complementation tests may be comparable with that secreted from its wild-type counterpart

NT1RE(pJK270) in these infection conditions. A previous study has shown that 5 μM (1.096 mg/L) to 35 μM (8 mg/L) of zeatin is secreted from *A. tumefaciens* C58 when the bacteria are grown at 28 °C for 24 h in the presence of 100–1000 μM AS (Powell *et al.*, 1988). The levels of *trans*-zeatin secreted from NT1RE(pJK270) when the bacteria were induced by 200 μM AS

at 19 °C for 40 h (Fig. 1B) were slightly lower than those expected if the strains were induced at 28 °C. Although different growth conditions indeed affect the amounts of cytokinin secretion from *A. tumefaciens*, the levels of *trans*-zeatin secretion during the *Agrobacterium* infection process *in planta* could not be estimated on the basis of the levels detected in AS-induced culture. Nevertheless, our data suggest that Tzs contributes to *Agrobacterium* virulence by promoting transformation efficiency during the early stages of infection. Future work will investigate an association of cytokinin concentration and transformation efficiency during *Agrobacterium* plant cultivation.

The discovery that Tzs interacts with VirB5 and its localization on the *Agrobacterium* cell surface (Aly *et al.*, 2008) and membrane (Lai *et al.*, 2006) suggests that Tzs may participate in the contact between host plant cells and bacterial cells. Tzs protein, an IPT, is generally believed to use substrates (i.e. HMBDP or DMAPP and AMP) in bacterial cytoplasm to synthesize *trans*-zeatin, which is then secreted into the culture medium (Krall *et al.*, 2002; Sakakibara, 2005, 2006; Sugawara *et al.*, 2008; Takei *et al.*, 2001). However, we cannot yet rule out that surface-localized Tzs may access substrates secreted from agrobacteria or infected plant cells to synthesize *trans*-zeatin directly in the extracellular space.

How do Tzs and its cytokinin product contribute to *Agrobacterium* virulence? One possible mechanism is that *trans*-zeatin secretion may stimulate plant cell division and growth at the infection sites at which plant cells become more susceptible to *Agrobacterium* infection. Alternatively, the accumulation of cytokinin at the infection sites by *A. tumefaciens* may create nutrient sinks at the wounded sites (Letham and Palni, 1983; Sakakibara, 2005, 2006; Walters and McRoberts, 2006), which may result in the suppression of host cell death and possibly facilitate bacterial establishment and growth.

Recent studies showing that plant hormones can affect both agrobacterial growth and *vir* gene expression (Anand *et al.*, 2008; Liu and Nester, 2006; Yuan *et al.*, 2007) also suggest a possible role of Tzs and cytokinin in agrobacterial physiology. Indoleacetic acid (IAA), the plant hormone auxin overproduced on T-DNA integration, inhibits bacterial growth and *vir* gene induction *in vitro* by competing with AS, the *vir* gene inducer (Liu and Nester, 2006). Exogenous application of the plant defence molecule salicylic acid (SA) to *Agrobacterium* cells inhibited the expression of *vir* genes including *tzs*, bacterial growth, bacterial attachment to plant cells and virulence (Anand *et al.*, 2008; Yuan *et al.*, 2007). On the basis of these studies, we examined whether the absence of Tzs protein and *trans*-zeatin secretion in *A. tumefaciens* may affect bacterial growth and *vir* gene expression under the AS-induced condition. The *tzs*-fs mutation caused a modest bacterial growth defect when induced by 200 µM AS at 19 °C up to 64 h (data not shown). However, growth of the *tzs*-fs mutant and wild-type bacteria was similar in medium lacking AS

(data not shown). In contrast with the effects of auxin and SA, which simultaneously inhibit agrobacterial growth and *vir* gene expression (Anand *et al.*, 2008; Liu and Nester, 2006; Yuan *et al.*, 2007), several *Vir* proteins indeed accumulated at higher levels in the *tzs*-fs mutant when grown in the same AS-induced condition as used for growth assays (data not shown). These results imply that Tzs and *trans*-zeatin may play a positive role in agrobacterial growth, but may negatively regulate *vir* gene expression in the AS-induced condition. The *tzs* mutant is still virulent, but whether this is a result of the higher expression of *Vir* proteins is unknown. Comprehensive and systematic analyses comparing the *tzs*-fs mutant and the wild-type in terms of bacterial growth, *vir* gene expression, bacterial attachment, cytokinin secretion and host cell responses during the infection process are required to elucidate the mechanistic role of Tzs in *Agrobacterium* virulence.

Intriguingly, our host range study revealed that the *tzs*-fs mutant enhanced tumorigenesis on green pepper and cowpea, whereas the same mutant caused reduced tumorigenesis on several plant species including *Arabidopsis*, white radish, potato, pai-tsai, loose leaf lettuce and carnation (Figs 1C, 2, 3 and 5; Table 1). These results suggest that Tzs may have additional roles that contribute to virulence on certain plant species. Further elucidation of the role of Tzs in the specificity of *Agrobacterium*–plant interactions might provide useful information for plant biotechnology applications.

EXPERIMENTAL PROCEDURES

Plasmid constructions

The bacterial strains and plasmids used in this study are listed in Table 2. The *A. tumefaciens* *tzs* frameshift mutant strain was generated by a double-crossover recombination using the suicide plasmid pJQ200KS (Quandt and Hynes, 1993). The plasmid pJQ200KS-*tzs*-fs, used to generate *tzs* frameshift mutants, was constructed by ligating *Xma*I/*Bam*HI-digested *Tzs*-fs1 polymerase chain reaction (PCR) product (927-bp DNA fragment containing the 3' end and sequences downstream of *tzs*) and the *Bam*HI/*Xba*I-digested *Tzs*-fs2 PCR product (703-bp DNA fragment containing sequences upstream of and the 5' region of *tzs*) into the *Xma*I/*Xba*I sites of the suicide vector pJQ200KS. The *Tzs*-fs1 PCR product was amplified with primers *Tzs*1-*Xma*I-F (5'-CCCCCGGGTAGGTGCCCGAGCCGTTCTT-3') and *Tzs*-fs-*Bam*HI-R (5'-GGACATGGGATCCAAATCGCAC-3'). The *Tzs*-fs2 PCR product was amplified with primers *Tzs*-fs-*Bam*HI-F (5'-GTGCGATTGGATCCCATGTCC-3') and *Tzs*2-*Xba*I-R (5'-GCTCTAGAAATGAAACACAGCCTCGGCA-3'). The resulting plasmid pJQ200KS-*tzs*-fs contained a single base-pair deletion in *tzs* obtained by introduction of a *Bam*HI site to create a frameshift mutation. The primer sequences of the designed restriction enzyme sites are

Table 2 Bacterial strains and plasmids.

Strains/plasmids	Relevant characteristics	References/sources
Strains		
<i>Agrobacterium tumefaciens</i>		
C58	Wild-type virulent strain containing nopaline-type Ti plasmid pTiC58	Lin and Kado, 1977
A136	C58 cured of its Ti plasmid pTiC58	Watson <i>et al.</i> , 1975
A208	A136 containing nopaline-type Ti plasmid pTiT37	Sciaky <i>et al.</i> , 1978
LBA4404	Ach5 strain containing a disarmed octopine-type Ti plasmid pAL4404	Hoekema <i>et al.</i> , 1983
EHA101	A136 strain containing a disarmed agropine-type Ti plasmid pEHA105	Hood <i>et al.</i> , 1986
NT1RE	Rm ^R Em ^R , C58 cured of its pTiC58	Watson <i>et al.</i> , 1975
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	Kao <i>et al.</i> , 1982
NT1RE(pJK270 <i>tzs</i> -fs)	Rm ^R Em ^R , Km ^R /Nm ^R , pJK270 containing <i>tzs</i> frameshift mutation	This study
<i>Escherichia coli</i>		
DH5α	Host for DNA cloning	Hanahan, 1983
S-17	Host for conjugation	Simon <i>et al.</i> , 1983
Plasmids		
pGEMT-Easy	Ap ^R , TA cloning vector	Promega, Madison, WI, USA
pUC19	Ap ^R , cloning vector	US Biochemical, Cleveland, OH, USA
pBluescript SK+	Ap ^R , cloning vector for blue/white screening	Stratagene, Cedar Creek, TX, USA
pCambia 2201	Cm ^R , a binary vector for plant transformation	Hajdukiewicz <i>et al.</i> , 1994, Hiei <i>et al.</i> , 1994
pCambia 2201-Gm	Cm ^R , Gm ^R , a binary vector for plant transformation	This study
pJQ200KS	Plasmid containing Gm ^R and <i>sacB</i> gene for selection of double crossover	Quandt and Hynes, 1993
pJQ200KS- <i>tzs</i> -fs	Gm ^R , pJQ200KS carrying <i>tzs</i> frameshift mutation	This study
pRU1064	Ap ^R , Tc ^R , a stable broad host range vector with IncP replicon	Karunakaran <i>et al.</i> , 2005
plncP-Tzs	Ap ^R , Tc ^R , a pRU1064-derived IncP plasmid carrying the promoter region and full length of <i>tzs</i> gene	This study
pEML771	Ap ^R , Tc ^R , an IncP plasmid with deletion of <i>tzs</i> open reading frame from plncP-Tzs	This study

Ap^R, Cm^R, Em^R, Gm^R, Km^R, Nm^R, Rm^R, Sp^R and Tc^R, resistant to ampicillin, chloramphenicol, erythromycin, gentamycin, kanamycin, neomycin, rifampicin, spectinomycin and tetracycline, respectively.

shown in bold and the primer sequences complementary to the specific region in the *A. tumefaciens* genome are shown in italics.

To generate the *A. tumefaciens* *tzs*-complemented strain, a 2.4-kbp genomic fragment of the *tzs* region (encompassing 927 bp upstream to 703 bp downstream of the *tzs* coding region) was amplified by PCR with the primers Tzs1-*Xma*I-F and Tzs2-*Xba*I-R. The *Xma*I/*Xba*I-digested PCR product was subsequently cloned into the same sites of pBluescript SK+, resulting in the plasmid pE453. The *Xba*I/*Kpn*I-digested 2.4-kbp *tzs* fragment from pE453 was cloned into the same sites of pUC19, thus generating the plasmid pE541. The 1.8-kbp *tzs* fragment was digested by *Hind*III from pE541 and was inserted at the same sites of an IncP plasmid pEML652 (Liu *et al.*, 2008) derived from the IncP broad host range vector pRU1064 (Karunakaran *et al.*, 2005; Prell *et al.*, 2002), thus resulting in the *tzs*-complemented construct plncP-Tzs. The plasmid plncP-Tzs was further digested by *Sac*I/*Pst*I to delete the *tzs* open reading frame (ORF), thus resulting in the plasmid pEML771 which served as a vector control.

For PCR, *A. tumefaciens* NT1RE(pJK270) cells grown overnight in 523 medium (10 g/L sucrose, 8 g/L casein enzymatic hydrolysate, 4 g/L yeast extract, 3 g/L K₂HPO₄, 0.3 g/L MgSO₄·7H₂O, pH 7.0), supplemented with the appropriate antibiotics, were used to isolate genomic DNA via a Qiagen genomic DNA kit (Qiagen

Inc., Valencia, CA, USA). The PCR product was amplified using the Advantage 2 PCR Enzyme System (BD Biosciences Clontech, Palo Alto, CA, USA) with 1 µg of genomic DNA as template. The PCR amplification cycles were performed as follows, unless indicated: 95 °C for 2 min (one cycle); 95 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min (35 cycles); and 72 °C for 10 min (one cycle). The plasmid constructs were confirmed by restriction mapping and DNA sequencing.

Generation of a *tzs* frameshift mutant

pJQ200KS-*tzs*-fs was used to generate a *tzs* frameshift mutation in *A. tumefaciens* NT1RE(pJK270) as described in Wu *et al.* (2008). To screen the *tzs* frameshift mutant, genomic colony PCR products amplified with primers Tzs-*Hind*III-R (5'-AAGCTT CCGAATTCGCGTCAGCGTGA-3') and Tzs2-*Xba*I-R were digested by *Bam*HI to confirm that the *Bam*HI site was created in the mutant, which was further confirmed by DNA sequencing.

Immunoblot and *trans*-zeatin analyses

Agrobacterium tumefaciens cells grown overnight in 523 medium (10 g/L sucrose, 8 g/L casein enzymatic hydrolysate, 4 g/L yeast extract, 3 g/L K₂HPO₄ and 0.3 g/L MgSO₄·7H₂O, pH

7.0) with the appropriate antibiotics were harvested (6000 *g*, 10 min) and resuspended in 20 mL of AB-MES medium [20 g/L glucose, 3 g/L K₂HPO₄, 0.3 g/L MgSO₄·7H₂O, 1 g/L NaH₂PO₄, 1 g/L NH₄Cl, 0.15 g/L KCl, 10 mg/L CaCl₂, 2.5 mg/L FeSO₄·7H₂O and 9.76 g/L 2-(*N*-morpholino)ethanesulphonic acid (MES), pH 5.5] without antibiotics at an optical density at 600 nm (OD₆₀₀) of 0.1. After growth at 28 °C to mid-logarithmic phase for 6 h, the bacterial cultures were further cultivated at 19 °C with or without 200 µM AS (Sigma-Aldrich, St. Louis, MO, USA), and the cells were collected at 40 h for further analysis after AS addition.

For immunoblot analysis, bacterial cells were harvested by centrifugation at 10 000 *g*, 4 °C for 10 min, resuspended in TE buffer [10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid (EDTA)] to OD₆₀₀ = 10, and subsequently disrupted by boiling for 10 min with sample loading buffer [0.0625 M Tris-HCl, 2.25% sodium dodecylsulphate (SDS) and 7.5% glycerol]. The total cell lysate was centrifuged at 10 000 *g* for 10 min to remove cell debris. The final protein concentrations were measured by a BCA protein assay (Pierce Biotechnology, Rockford, IL, USA) with a Power Wave X 340 Microplate Reader (Bio-Tek Instruments Inc., Winooski, VT, USA). Equal amounts (15 µg) of total protein extract were resolved by electrophoresis through 16.5% Tricine SDS-polyacrylamide gels (Schagger and von Jagow, 1987) and transferred to Hybond ECL nitrocellulose membranes (Amersham Biosciences, Uppsala, Sweden). Immunoblot analyses were performed with a Tzs antibody (1 : 5000 or 1 : 10 000 dilution) (Aly *et al.*, 2008; Krall *et al.*, 2002) or with neomycin phosphotransferase II (NptII, encoded by *Tn5* inserted in pJK270) antibody (1 : 50 000 dilution) (Sigma-Aldrich) as an internal control, followed by incubation with goat anti-rabbit immunoglobulin G (IgG) conjugated with horseradish peroxidase (HRP) (1 : 50 000 dilution) (Chemicon International, Inc., Temecula, CA, USA). The membranes were developed by a chemiluminescent detection method (PerkinElmer Life and Analytical Sciences, Inc., Boston, MA, USA).

To detect *trans*-zeatin by UPLC, AS-induced bacterial cultures grown at 19 °C for 40 h in AB-MES (pH 5.5) were harvested by centrifugation at 13 000 *g* for 15 min. In total, 1 mL of the supernatant fluid was filtered through a 0.2-µm membrane to remove contaminating bacterial cells. For each analysis, 5 µL of *trans*-zeatin (Sigma-Aldrich) standard (0.01, 0.1, 1 and 10 mg/L) or filtered supernatant was subjected to a Waters Acquity UPLC system (Milford, MA, USA) by fractionation through an Acquity UPLC BEH C₁₈ column (1.7 µm, 2.1 × 50 mm; Waters). The column was equilibrated in 0.2 M acetic acid (pH 3.2) and eluted with a linear gradient of methanol (1%–99% over 3 min, 99% for 0.5 min, 99%–1% over 0.1 min, 1% for 0.4 min). Samples were detected with a UV–visible photodiode array detector at 265 nm. The *trans*-zeatin concentration was determined on the basis of the peak area and calculated according to the standard curve obtained from the standards.

Tumorigenesis and transient transformation assays

The *Arabidopsis* root transformation assays (Hwang and Gelvin, 2004; Nam *et al.*, 1997, 1998, 1999; Zhu *et al.*, 2003) and the quantitative tumorigenesis assay on potato tuber discs (Shurvinton and Ream, 1991; Wu *et al.*, 2008) were performed as described previously. All *A. tumefaciens* strains were grown in 523 medium supplemented with appropriate antibiotics (erythromycin, 50 µg/mL; rifampicin, 50 µg/mL; gentamycin, 50 µg/mL; kanamycin, 20 µg/mL) at 28 °C and to OD₆₀₀ = 0.8–1.0. The bacterial cells were washed with 0.9% sodium chloride and resuspended in 0.9% sodium chloride at the desired bacterial concentrations for infection. For transient transformation assays on *Arabidopsis* roots, root segments were infected with each *A. tumefaciens* strain containing the binary vector pCambia2201-Gm. A gentamycin-resistance gene cassette was cloned as a *Bam*HI-*Eco*RI fragment into the same sites of pCambia2201 (Hajdukiewicz *et al.*, 1994; Hiei *et al.*, 1994), resulting in the plasmid pCambia2201-Gm. For both stable and transient transformation of *Arabidopsis* roots, at least 10 different 3–4-week-old *Arabidopsis thaliana* plants (ecotype: Ws) were infected with 100 µL of each *A. tumefaciens* strain and co-cultivated on MS medium with or without cytokinin in the medium at 22–24 °C for 2 days. More than 60 root segments were examined for each plant for each independent transformation assay. For transient transformation assays, 7 days after infection, the *Arabidopsis* root segments were stained with 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc) staining solution (50 mM sodium phosphate buffer, pH 7.0; 0.1% Tween 20, 3% sucrose and 1–2 mM X-gluc) for 1 day at 37 °C. Roots were then examined using a stereoscopic microscope to obtain transient transformation efficiencies. For tumour assays on potato tuber discs, the discs were infected with 10 µL of bacterial cultures and co-incubated for 2 days. The tumours were scored after 5 weeks, and at least 60 potato discs were infected with each *A. tumefaciens* strain for each independent transformation assay. The tumour assays on other plant species were performed as described previously (Palumbo *et al.*, 1998) with minor modification. Stems of 3–4-week-old plants were injected with 100 µL of 10⁹ cfu/mL bacterial cultures using a syringe. After inoculation, the plants were grown in a glasshouse for 5–7 weeks before scoring the tumours. At least 15 different plants were infected with each *A. tumefaciens* strain for each independent transformation assay. The strain NT1RE (no Ti plasmid) was tested as a negative control in each tumour assay experiment.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 Summary of tumour assay results for various *Agrobacterium* strains on white radish stems.

Table S2 Tumour assay results of the wild-type *Agrobacterium* strain NT1RE(pJK270), *tzs-fs* mutant and *tzs*-complemented strain on *Arabidopsis* roots.

Table S3 Transient transformation assay results of the wild-type *Agrobacterium* strain NT1RE(pJK270), the *tzs-fs* mutant and the *tzs*-complemented strain on *Arabidopsis* roots.

Table S4 Tumour assay results of the wild-type *Agrobacterium* strain NT1RE(pJK270) and the *tzs-fs* mutant on potato tuber discs.

Table S5 Tumour assay results of the wild-type *Agrobacterium* strain NT1RE(pJK270) and the *tzs-fs* mutant on various plant species.

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