Biological Activity of the *tzs* Gene of Nopaline *Agrobacterium tumefaciens* GV3101 in Plant Regeneration and Genetic Transformation

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Agrobacterium tumefaciens has been widely used in plant genetic transformation. Hormone-encoding genes residing in the T-DNA region have been removed, resulting in disarmed *Agrobacterium* strains that are used in various transformation experiments. Nopaline *Agrobacterium* strains, however, carry another hormone gene, *trans*-zeatin synthesizing (*tzs*), that codes for *trans*-zeatin in the virulence region of the tumor-inducing plasmids. We investigated the activity and function of the *tzs* gene of a nopaline *Agrobacterium* sp. strain GV3101 in plant in vitro regeneration. Leaf explants of tobacco and *Nicotiana benthamiana* co-cultured with strain GV3101 exhibited active shoot regeneration in media without added plant growth regulators. On medium without plant growth regulators, transgenic shoots were also induced from explants co-cultured with GV3101 containing a binary vector. Enzyme-linked immunosorbent assay showed that cell-free extracts of *Agrobacterium* sp. strain GV3101 culture contained the *trans*-zeatin at 860 ng/liter. Polymerase chain reaction using *tzs*-specific primers showed that the *tzs* gene was present in strain GV3101 but not in other *Agrobacterium* strains. The study showed that the *tzs* gene in GV3101 was actively expressed, and that *trans*-zeatin produced in the *Agrobacterium* strain can promote plant shoot regeneration.

Agrobacterium tumefaciens is a plant pathogen which can cause gall disease in plant tissues (Braun 1958; De Cleene and De Ley 1976; Zhu et al. 2000). *Agrobacterium* spp. can transfer the transferred DNA (T-DNA) region of its tumor-inducing (Ti) plasmid into plant cells at the infected wound sites and the T-DNA can then be incorporated into the plant genome. Gall formation is due to the expression of oncogenic genes located in the T-DNA region (Akiyoshi et al. 1987; Citovsky et al. 2007; Gelvin 2003; Jameson 2000; Otten et al. 1999; Shaw 1995). If the oncogenes coding for hormones are removed from the T-DNA region, the result is a disarmed *Agrobacterium* sp. (Hood et al. 1986; Ooms et al. 1982; Mankin et al. 2007; Palanichelvam et al. 2000; Zhan et al. 1990). When the

oncogenes are replaced with genes of interest, these target genes can be transferred into the plant genome. *Agrobacterium*-mediated plant transformation has now been widely used in molecular biology research and biotechnology for introduction of foreign genes into plant cells. Different *Agrobacterium* strains that have different features have been developed and used in plant genetic transformation (Hoekema et al. 1984; Holsters et al. 1980; Hood et al. 1993; Hooykaas and Schilperoort 1992; Hu and Du 2006; Pitzschke and Hirt 2010; Tzfira et al. 2004; Wood et al. 2001).

T-DNA transfer is not regulated by T-DNA itself but is actually mediated by a series of virulence (*vir*) genes located in the *vir* region of the Ti plasmid (Cascales and Christie 2004; Christie et al. 2005; Hoekema et al. 1983; Judd et al. 2005; Lacroix and Citovsky 2011; Ziemienowicz et al. 2001). In nopaline-type *Agrobacterium* strains such as GV3101, the *vir* region contains a specific gene, *trans*-zeatin synthesizing (*tzs*), that codes for *trans*-zeatin synthesis (TZS) (Akiyoshi et al. 1985; Beaty et al. 1986; Jameson 2000; Krall et al. 2002). Zeatin is a cytokinin, a main type of phytohormone that can promote cell differentiation and growth (Hwang et al. 2010; Mok and Mok 2001; Sakakibara 2006; Takei et al. 2001). The TZS catalyzes the last step of the biosynthesis of the *trans*-zeatin ribosides in *Agrobacterium* (John and Amasino 1988; Krall et al. 2002; Powell and Morris 1986; Powell et al. 1988). Because the *tzs* gene is not transferred into plant cells and, therefore, does not cause gall disease in plant, this gene has been retained intact in nopaline *Agrobacterium* strains used in plant transformation.

The biological function of *tzs* is proposed to stimulate plant cell growth upon infection of *Agrobacterium* spp. to plant tissues. This stimulation may increase the efficiency of T-DNA transfer into plant cells (Akiyoshi et al. 1987; Beaty et al. 1986; Heinemeyer et al. 1987; Hwang et al. 2010; John and Amasino 1988; Powell et al. 1988). However, thus far, there is no direct evidence for this role during the *Agrobacterium*-plant interaction. Because *tzs* does not exist in all *Agrobacterium* strains, the gene is not considered to be essential for virulence and the *tzs*-containing *Agrobacterium* strains do not necessarily generate higher transformation efficiency.

Plant genetic transformation with *Agrobacterium* spp. is usually conducted with tissue-cultured plant materials, and plant growth regulators (PGR) are normally added into the culture media to promote cell regeneration. We investigated the biological activity of the *tzs* gene of nopaline *Agrobacterium* sp. strain GV3101 in plant in vitro culture.

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RESULTS

Agrobacterium sp. strain GV3101 strain promotes plant cell regeneration.

Leaf explants of tobacco and *Nicotiana benthamiana* were inoculated with *Agrobacterium* sp. strain GV3101 and then cultured on Murashige and Skoog (MS) medium without adding PGR; specifically, cytokinins. As much as 86% of tobacco explants showed in vitro shoot regeneration (Table 1) in hormone-free medium. Similarly, 50% of the *N. benthamiana* explants exhibited vigorous shoot regeneration on medium without PGR (Table 1). No shoot regeneration was observed on explants placed on PGR-free medium and without GV3101 inoculation (Table 1; Fig. 1).

Regeneration with supernatant of GV3101 culture.

Regeneration of explants inoculated with GV3101 indicated that the bacterium-secreted hormones might have stimulated cell regeneration. Explants inoculated in the supernatant of GV3101 culture for different time lengths and then cultured in PGR-free medium containing timentin to control bacterial growth exhibited shoot regeneration (Fig. 2). It appeared that an 8-h treatment of the explants with supernatant was sufficient to promote shoot regeneration. The regeneration rate increased significantly when the explants were cultured for longer periods of time (Fig. 2).

Evaluation of different *Agrobacterium* strains on plant regeneration.

GV3101 belongs to the nopaline *Agrobacterium* group in which the *vir* region carries the *tzs* gene. Regeneration of explants co-cultured with GV3101 indicated that the *tzs* gene in the strain might be responsible for the regeneration; therefore, strains without the *tzs* gene should not show a shoot induction effect. To test this, we compared strain GV3101 with three *Agrobacterium* strains without the *tzs* gene—namely, LBA4404 (Hoekema et al. 1983), EHA101, and EHA105 (Hood et al. 1993)—on plant regeneration. Tobacco leaf explants were co-cultured with these strains and then placed on PGR-free MS medium. Up to 97.5% of tobacco explants co-cultured

with GV3101 produced shoots (Table 2; Fig. 1B) but no shoot development was observed on explants cultured with the other *Agrobacterium* strains (Table 2; Fig. 1B). The results also showed that acetosyringone had no effect on shoot production (Table 2; Fig. 1B).

Plant transformation without using PGR.

Based on the regeneration promotion by GV3101, we then speculated that this *Agrobacterium* strain might also promote transgenic cell regeneration on medium without PGR. Leaf explants of tobacco and *N. benthamiana* co-cultured with *Agrobacterium* sp. strain GV3101 containing the binary vector tCUP- β -glucuronidase (GUS) were placed on a PGR-free medium containing kanamycin, which would inhibit the regeneration of untransformed cells. Although control leaf discs that were not exposed to the *Agrobacterium* strain did not produce any shoots in the presence of kanamycin, leaf explants inoculated with GV3101/tCUP-GUS exhibited shoot development (Table 3). The transformation was confirmed by histochemical analysis of the GUS expression and polymerase chain reaction (PCR) analysis using *nptII*-specific primers (data not shown) using standard methods well established in our laboratory (Tian et al. 2002, 2003).

Quantification of *trans*-zeatin in the *Agrobacterium* culture medium.

Different *Agrobacterium* strains were cultured on Luria-Bertani (LB) medium containing appropriate antibiotics (Table 4). The supernatant of each *Agrobacterium* strain was purified and *trans*-zeatin production was quantified using enzyme-linked immunosorbent assay (ELISA). As expected, a significant amount (860 ng/liter) of *trans*-zeatin was detected in GV3101 culture (Table 5) but no or little *trans*-zeatin was detected in the media cultured with other *Agrobacterium* strains.

Analysis of the presence of the *tzs* gene in *Agrobacterium* strains.

DNA primers specific to the *tzs* gene were designed and used for PCR analysis to detect the presence of the *tzs* gene in different *Agrobacterium* strains. A 664-bp DNA fragment cor-

Table 1. Shoot regeneration of tobacco and *Nicotiana benthamiana* leaf explants incubated with *Agrobacterium* sp. strain GV3101 on plant growth regulator (PGR)-free medium

Plant	Culture condition	Number of explants	Response (%)	Number of shoots/explant $\pm$ SE ^y
Tobacco	No PGR ^z	50	0	0
	GV3101	48	86.00	3.65 $\pm$ 0.34
<i>N. benthamiana</i>	No PGR ^z	50	0	0
	GV3101	50	50.00	1.32 $\pm$ 0.27

^y SE = standard error of the mean.

^z No PGR added.

Table 2. Shoot regeneration of tobacco leaf explants co-cultured with different *Agrobacterium* strains with or without acetosyringone on plant growth regulator-free medium

<i>Agrobacterium</i> strain	Acetosyringone	Number of explants	Response (%)	Number of shoots/explant mean $\pm$ SE ^z
No	–	30	0	0
	+	40	0	0
GV3101	–	39	97.50	3.59 $\pm$ 0.50 a
	+	40	97.51	3.83 $\pm$ 0.38 a
LBA4404	–	30	0	0
	+	40	0	0
EHA101	–	40	0	0
	+	40	0	0
EHA105	–	40	0	0
	+	40	0	0

^z SE = standard error of the mean. Means followed by the same letter are not significantly different ($P < 0.05$) according to Tukey's honestly significant difference test.

responding to the *tzs* gene was amplified from GV3101. No *tzs* gene was detected in other *Agrobacterium* strains (Fig. 3).

Plant development and recovery.

Induced shoots of tobacco and *N. benthamiana* produced following *Agrobacterium* sp. strain GV3101 inoculation showed normal growth and development. Shoots removed from explants and put into fresh PGR-free medium developed roots. Plantlets further developed in Magenta boxes (Fig. 1Ae and f) prior to transplanting into soil. Normal and full plants were recovered in a greenhouse (Fig. 1Ag and h).

DISCUSSION

The *tzs* gene in nopaline-type *Agrobacterium* strains has long been documented (Barry et al. 1984; Beatty et al. 1986). Various studies have been conducted to characterize this gene and reveal the potential biological functions of *Agrobacterium*

spp. in the natural situation (Aly et al. 2008; John and Amasino 1988; Krall et al. 2002; Lee and Gelvin 2008; Powell et al. 1988). Disarmed nopaline *Agrobacterium* strains have been widely used in plant biotechnology, especially for plant genetic transformation using in vitro culture. In a study on plant transformation, Fillatti and associates (1987) report that using a nopaline *Agrobacterium* sp. strain C58 could enhance the regeneration rate of a poplar hybrid clone in medium where PGR were supplied. They suggested that the *tzs* gene in the *vir* region might provide a certain hormone environment that stimulated plant regeneration. However, a systemic study to explore the possible function of the *tzs* gene with respect to plant regeneration has not previously been reported.

Shoot induction from leaf explants co-cultured with *Agrobacterium* sp. strain GV3101 was significant. The supernatant of the GV3101 culture also promoted active shoot regeneration. These results indicated that this *Agrobacterium* strain secreted plant hormones or hormone-like compounds that stimulated

Fig. 1. Plant in vitro regeneration via interactions with different *Agrobacterium* strains. **A**, Shoot induction and plant development of tobacco (b, e, and g) and *Nicotiana benthamiana* (d, f, and h) inoculated with *Agrobacterium* GV3101. a, Tobacco control and c, *N. benthamiana* control. **B**, Shoot induction of tobacco and *N. benthamiana* inoculated with *Agrobacterium* strains GV3101 (a and e), LBA4404 (b and f), EHA101 (c and g), and EHA105 (d and h) on plant growth regulator-free medium.

cell differentiation and regeneration. Strain GV3101 contains a *tzs* gene (John and Amasino 1988; McKenzie et al. 1994; Powell and Morris 1986) that might be actively producing *trans*-zeatin, a type of phytohormone. To confirm that in vitro shoot induction was due to the activity of *tzs* gene in GV3101, three *Agrobacterium* strains, including LBA4404, EHA101, and EHA105,

that do not belong to the nopaline group and do not contain the *tzs* gene in the Ti plasmid, were evaluated for plant shoot initiation. As expected, shoot regeneration was only observed in explants inoculated with GV3101 and no regeneration was observed with any other strains not belonging to the nopaline group. This further indicated that the observed shoot regen-

Fig. 2. Shoot induction from tobacco leaf explants inoculated with the supernatant of *Agrobacterium* GV3101 for different time lengths on plant growth regulator-free medium.

Table 3. Transgenic shoot development of tobacco and *Nicotiana benthamiana* leaf explants co-cultured with *Agrobacterium* sp. strain GV3101 on plant growth regulator-free medium

Plant	Culture condition	Number of explants	Response (%)	Number of shoots/explant $\pm$ SE ^z
Tobacco	Control	30	0	0
	GV3101/tCUP	50	20.00 $\pm$ 1.47	0.22 $\pm$ 0.07
<i>N. benthamiana</i>	Control	30	0	0
	GV3101/tCUP	50	16.3 $\pm$ 3.42	0.21 $\pm$ 0.04

^z SE = standard error of the mean.

Table 4. Disarmed *Agrobacterium tumefaciens* strains used in the study

Strain name	Chromosomal background	Ti plasmid derivation ^y	Alkaloids-type	Antibiotic resistance ^z	Reference
GV3101	C58	pTiC58	Nopaline	Rif, gen	Koncz and Schell 1986
LBA4404	Ach5	pTiAch5	Octopine	Rif	Ooms et al. 1982
EHA101	C58	pTiBo542	Succinamopine	Rif, km	Hood et al. 1986
EHA105	C58	pTiBo542	Succinamopine	Rif	Hood et al. 1993

^y Ti = tumor inducing.

^z Rif = rifampicin, gen = gentamycin, and km = kanamycin.

Table 5. Levels of *trans*-zeatin in culture medium inoculated with different *Agrobacterium* strains

	<i>Agrobacterium</i> strain				
	GV3101-blank	GV3101-tCUP	LBA4404	EHA101	EHA105
<i>Trans</i> -zeatin (ng/liter) ^z	860.14 $\pm$ 3.58	877.85 $\pm$ 2.66	4.75	<1	<1

^z Data shown $\pm$ standard error of the mean.

Fig. 3. Polymerase chain reaction analysis of *trans*-zeatin synthesizing (*tzs*) gene in different *Agrobacterium* strains. **A**, Lane 1, marker; lane 2, negative control; lane 3, blank GV3101; lanes 4 to 6, GV3101 containing different binary vectors. **B**, Lane 1, GV3101; lane 2, LBA4404; lane 3, EHA101; lane 4, EHA101; lane 5, negative control; lane 6, marker.

eration resulted from the activity of the *tzs* gene in strain GV3101.

Expression of the *tzs* gene in *Agrobacterium* strains can be induced by acetosyringone (Anand et al. 2008; Beaty et al. 1986; Cho and Winans 2005; Powell et al. 1988; Sheikholslam and Weeks 1987; Stachel et al. 1985; Yuan et al. 2007). However, no significant difference in shoot induction was observed in the presence or absence of acetosyringone in this study. Cytokinin production by GV3101 without acetosyringone might be adequate for plant cell regeneration and the incremental effect of acetosyringone was probably minimal in this situation. No shoot induction was observed in explants in the presence of acetosyringone but without GV3101 inoculation. Similar results were also observed using other *Agrobacterium* strains. Thus, in vitro regeneration of explants co-cultured with GV3101 was not due to acetosyringone.

Except for the flower dip method in *Arabidopsis* (Bechtold et al. 1993; Hooykaas and Schilperoort 1992; Zhang et al. 2006), plant genetic transformation is usually conducted by inducing in vitro regeneration with PGR being added to the culture media. We speculated that GV3101 could also promote transgenic cell in vitro regeneration. Genetic transformations conducted with GV3101 containing the tCUP-GUS vector (Foster et al. 1999; Tian et al. 2002, 2003) resulted in efficient transgenic shoot development with both tobacco and *N. benthamiana*. *Trans*-zeatin produced by GV3101 apparently also promoted transgenic cell differentiation for shoot development. Based on this observation, *trans*-zeatin produced by GV3101 may have partially contributed to the transgenic cell regeneration in various reported transformation experiments in which cytokinin was still added in culture media.

It was proposed that gene *tzs* might stimulate T-DNA transfer into plant cells (Guo et al. 2005; John and Amasino 1988; Mankin et al. 2007; Vergunst et al. 2005). In a recent study by Hwang and associates (2010), the loss of TZS protein expression 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. These data suggest that TZS may contribute to both gene transfer and cell regeneration. Because TZS is not produced by all *Agrobacterium* strains, the *tzs* gene is not considered to be essential for T-DNA transfer. The *tzs* gene may be a host range factor contributing to *Agrobacterium* infection in certain plants (Aly et al. 2008; Hwang et al. 2010; Hooykaas and Schilperoort 1992; Zupan et al. 2000). Further research is needed to investigate the involvement of the *tzs* gene in plant genetic transformation.

A molecular biology approach was applied to analyze the *tzs* gene in *Agrobacterium* strains. The presence of the *tzs* gene in GV3101 was confirmed by PCR using *tzs*-specific primers. The *tzs* gene was not detected in other *Agrobacterium* strains that do not belong to nopaline group. These results are in agreement with earlier studies that showed that the *tzs* gene was present only in nopaline *Agrobacterium* strains (Akiyoshi et al. 1985; Beaty et al. 1986; Regier and Morris 1982; Veselov et al. 2003). A significant production of *trans*-zeatin by GV3101 was determined by ELISA, indicating that zeatin produced in GV3101 culture contributed to shoot in vitro regeneration observed in the study. In a recent study, transgenic poplars containing the *tzs* gene controlled by the 35S promoter showed high levels of cellular zeatin, and various plant development phenotypes associated with cytokinin-mediated morphogenesis were observed (Choi et al. 2009). When the isopentenyl transferase (*ipt*) gene from *Agrobacterium* was introduced into kiwifruit, the content of a *trans*-zeatin precursor increased in the transgenic line, indi-

cating that morphological changes were related to expression of the *ipt* gene (Honda et al. 2011). Similarly, *trans*-zeatin produced by the expression of the *tzs* gene had the biological function and, indeed, stimulated cell division and growth.

This study, for the first time, reports that the *tzs* gene in a nopaline *Agrobacterium* strain actively expressed and produced a significant amount of *trans*-zeatin. The hormone produced showed biological function, induced plant cell differentiation, and promoted in vitro shoot development normally.

MATERIALS AND METHODS

Plant materials.

The donor plants of tobacco (*N. tabacum*) 'SR1' and *N. benthamiana* were maintained in vitro on MS medium (Murashige and Skoog 1962) in Magenta containers. The plants were propagated by subculturing the plant notes onto fresh medium every 2 months and were maintained at 25°C in white light at approximately 54 to 72 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with light and dark photo-periods of 16 and 8 h, respectively.

Agrobacterium strain preparation.

Four *A. tumefaciens* strains were used in this research (Table 4). Strain GV3101/PMP90 was obtained from B. Miki's laboratory collection, Agriculture and Agri-Food Canada, Ottawa. LBA4404 was purchased from Invitrogen Inc. EHA101 and EHA105 were kindly provided by R. Scorza (United States Department of Agriculture Appalachian Fruit Research Station Kearneysville, WV, U.S.A.). *Agrobacterium* strains were grown in liquid LB medium (Miller 1972) containing appropriate antibiotics (Table 4) at 28°C to mid-log phase (with an optical density at 600 nm [OD₆₀₀] of approximately 1). The culture was diluted 1:1 with LB medium for plant inoculation.

The tCUP-GUS (Foster et al. 1999) was cloned into pRD400 as described before (Tian et al. 2003). The construct was introduced into *A. tumefaciens* GV3101/pMP90 by electroporation and cultured in LB medium with appropriate antibiotics.

Plant explant inoculation.

Plant leaves were cut into 0.5-cm² pieces in a laminar hood. The explants were dipped in *Agrobacterium* culture solution for 15 min and then placed on sterile filter paper to remove excess *Agrobacterium* solution. Explants were cultured on MS salts (Murashige and Skoog 1962), B5 vitamins (Gamborg et al. 1968), 3% sucrose, and 0.25% Gelrite, pH 5.7, as described by Tian and associates (2002) but without PGR. After co-culturing for 2 days, explants were placed on PGR-free MS medium with timentin (300 mg/liter) to inhibit *Agrobacterium* growth and the culture was maintained under the conditions described previously.

Co-culture with supernatant of GV3101.

The *Agrobacterium* sp. strain GV3101 culture was cultured until the OD₆₀₀ reached approximately 1. The culture was centrifuged for 15 min at 5,000 rpm and the supernatant was collected. Explants were dipped in the supernatant for 15 min and then placed on PGR-free MS medium. After 8, 24, and 48 h, explants were transferred to PGR-free MS medium containing timentin.

Plant genetic transformation.

Tobacco and *N. benthamiana* leaf explants were co-cultured with GV3101 containing tCUP-GUS binary vector (Foster et al. 1999; Tian et al. 2003). Explants were then maintained on MS medium containing timentin (300 mg/liter) and kanamycin (100 mg/liter). The transformation followed the procedure described previously (Tian et al. 2002, 2003).

PCR analysis of the *tzs* gene in different *Agrobacterium* strains.

To analyze the presence of the *tzs* gene in different *Agrobacterium* strains, a PCR was performed using *tzs*-specific primers. The primers used (forward primer: 5'-TCTGGCCACTGAGG AAAATC-3' and reverse primer: 5'-ATCTACGGACCGACTT GCAG-3') were designed online by software primer 5 and the specificity of the primer was verified online by BLAST of National Center for Biotechnology Information. The expected length of the PCR product was 664 bp.

Agrobacterium cultures were grown and prepared as described earlier. A single bacterial colony was added to the PCR reaction solution as the template for each PCR analysis. The PCR reaction contained 2 µl of 10× buffer, 1 µl of primer mix, 0.4 µl of dNTP (10×), 16.4 µl of water, and 0.2 µl of *Taq* DNA polymerase (Qiagen, Basel, Switzerland U.S.A.). DNA amplification reactions were performed in a thermocycler (model mastercycler gradient; Eppendorf Canada Ltd., Mississauga, Ontario, Canada) with the following conditions: 1 cycle at 94°C for 5 min; 35 cycles at 94°C for 30 s, 58°C for 1 min, and 72°C for 1 min; and a final step of 72°C for 4 min. Amplification products were separated by 1.2% agarose gel electrophoresis, stained with ethidium bromide, and photographed.

Quantification of *trans*-zeatin in the bacterium culture medium.

The quantification of *trans*-zeatin was conducted by ELISA. All strains of *Agrobacterium* were grown in 500 ml of LB medium containing appropriate antibiotics to mid-log phase (with OD₆₀₀ at approximately 1). Samples were extracted with 80% methanol for 12 h at 4°C in the dark. The homogenate was filtered and methanol fractions of the extracts were passed through a C₁₈ Sep-Pak cartridge (Waters, Milford, MA, U.S.A.). Eluates were evaporated in vacuum at 40°C to leave an aqueous phase which was then diluted with 9 volumes of 40 mM ammonium acetate (pH 6.5). Diethylaminoethyl (DEAE) equilibrium cellulose (4 mg) in 40 mM ammonium acetate (pH 6.5) was added and the mixture was shaken for 10 min (Banowetz 1992). After centrifugation to remove the DEAE cellulose, the supernatant was passed through a C₁₈ Sep-Pak cartridge, which was then washed with 0.1 M acetic acid and eluted with 6 ml 25% methanol in 0.1 M acetic acid (vol/vol). After being concentrated with PEG2000 to a volume of 0.5 ml, *trans*-zeatin was quantified by ELISA. The amount of *trans*-zeatin was calculated by a commercial immunoassay kit for *trans*-zeatin ribosides (Phytodetek; AgDia Inc., Elkhart, IN, U.S.A.). ELISAs were performed according to the manufacturer's protocol.

Statistical analysis.

Data were analyzed using SPSS 16.0.2 computer statistical program (IBM SPSS, Armonk, NY, U.S.A.) and treatment means were compared using Tukey's honestly significant difference test at the probability of 5%.

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