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EFFECT OF AN ENHANCED CAMV 35S PROMOTER AND A FRUIT-SPECIFIC PROMOTER ON *UIDA* GENE EXPRESSION IN TRANSGENIC TOMATO PLANTS

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Summary

Two different promoters, a cauliflower mosaic virus (CaMV) 35S promoter with a 5′- untranslated leader sequence from alfalfa mosaic virus RNA4 (designated as CaMV 35S/AMV) and an E-8 fruit-ripening-specific promoter, were compared to evaluate their effects on expression of the *uidA* reporter gene in transgenic tomato plants. In order to generate sufficient numbers of transgenic tomato plants, both a reliable regeneration system and an efficient *Agrobacterium* transformation protocol were developed using 8-d-old cotyledons of tomato (*Lycopersicon ecsulentum* Mill. cv. Swifty Belle). Two sets of constructs, both derivatives of the binary vector pBI121, were used in transformation of tomato whereby the *uidA* gene was driven either by the CaMV 35S/AMV or the E-8 fruit-ripening-specific promoter. Southern blot hybridization confirmed the stable integration of the chimeric *uidA* gene into the tomato genome. Fruit and leaf tissues were collected from T_0 and T_1 plants, and assayed for β-glucuronidase (GUS) enzyme activity. As expected, both vegetative and fruit tissues of transgenic plants carrying the *uidA* gene under the control of CaMV 35S/AMV showed varying levels of GUS activity, while no expression was observed in vegetative tissues of transgenic plants carrying the *uidA* gene driven by the E-8 promoter. All fruits from transgenic plants produced with both sets of constructs displayed expression of the *uidA* gene. However, when this reporter gene was driven by the CaMV 35S/AMV, GUS activity levels were significantly higher than when it was driven by the E-8 fruit-specific promoter. The presence/absence of the *uidA* gene in T_1 plants segregated in a 3:1 Mendelian ratio.

Key words: E-8 fruit-specific promoter; AMV leader sequence; β-glucuronidase (GUS); *Agrobacterium* transformation; plant regeneration.

Introduction

Several approaches to introduce foreign DNA into tomato (*Lycopersicon esculentum* Mill.) using *Agrobacterium*-mediated transformation (McCormick et al., 1986; Chyi and Phillips, 1987; Filatti et al., 1987; Davis et al., 1991; Joao and Brown, 1993; Hamza and Chupeau, 1993; Liu et al., 1995) and microprojectile bombardment (Van Eck et al., 1995) have been reported. In these transformation studies, most constructs contained a *uidA* reporter gene (Jefferson et al., 1986) driven by a CaMV 35S promoter allowing detection of transient and eventually stable expression of the *uidA* gene in transgenic plants, regardless of the stage of plant development or tissue localization. Several fruit-ripening-specific promoters such as E-4, E-8, and 2A11 were identified in tomato. They have been cloned, characterized, and studied in relation to the effect of ethylene on fruit ripening (Van Haaren and Houck, 1993; Nicholass et al., 1995; Xu et al., 1996; Deikman et al., 1998). These fruit-specific promoters have been mostly used to investigate the role of ethylene in fruit ripening (Coupe and Deikman, 1997; Deikman et al., 1998). However, recently, we have evaluated the use of the E-8 and CaMV 35S promoters in driving the expression of an antigenic F-protein gene from the human respiratory syncytial virus (RSV) in transgenic tomato fruit in our effort to develop an edible oral vaccine against this viral disease (Sandhu et al., 2000). Although the level of the F-antigenic protein gene in fruit of different transgenic tomato plants was highest in a transgenic line carrying the RSV-F protein gene driven by the E-8 promoter, we were still hopeful that we can further increase the level of the recombinant protein by manipulating the promoter driving the gene. In a previous study, we demonstrated that the insertion of a 5′-untranslated leader sequence from alfalfa mosaic virus (AMV) between the CaMV 35S promoter and the RSV-F gene increased the level of gene expression in apple leaf protoplasts by 5.5-fold (Sandhu et al., 1999). Therefore, in this present study, we were interested in comparing the effects of an enhanced CaMV 35S promoter with that of the E-8 fruit-ripening-specific promoter on the level of gene expression in tomato fruits. In order to demonstrate the usefulness of this comparative promoter study to a wide group of transgenes, we elected to use the *uidA* reporter as a model gene system for quantitative and qualitative analyses of gene expression in transgenic tomato plants.

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Materials and Methods

Plant material. Seeds of tomato (*L. esculentum* cv. Swifty Belle), obtained from Stokes Seeds, Inc. (Buffalo, NY), were sterilized with 20% Clorox® commercial bleach (0.105% sodium hypochlorite) for 15 min, rinsed three times in sterilized distilled water (5 min per rinse), and allowed to germinate in glass jars containing full-strength MS salts (Murashige and Skoog, 1962), B_5 vitamins (Gamborg et al., 1968), and 3% sucrose. The medium was solidified with 7 g l^{-1} agar (PhytoTechnology Lab., cat. no. A296, Shawnee Mission, KS), and the pH was adjusted to 5.7 with 1.0 *N* NaOH prior to autoclaving.

Evaluation of effect of different media formulations on shoot organogenesis. Cotyledonary explants from 8-d-old seedlings were incubated on different regeneration media to identify the best formulation for inducing shoot organogenesis. Compositions of these different media are listed in Table 1. These various formulations included two published tomato regeneration (TR) media (Hamza and Chupeau, 1993; Van Roekel et al., 1993), designated TR-1 and TR-2, and six new modifications of these media designated TR-3 to TR-8. The pH of each of the media was adjusted prior to autoclaving to either 5.7 or 5.8 with 1.0 *N* NaOH. A total of 16–18 explants per plate were cultured, three plates per treatment were used in a completely randomized block design, and the whole experiment was repeated once.

As all explants in all treatments produced shoots, efficiency of shoot regeneration was based on the number of shoots regenerated per explant after 3 wk of culture. Data were analyzed using the SAS statistical analysis package, and mean comparisons were made using least significant difference at the 0.05 probability level.

Agrobacterium *strain and binary vector. Agrobacterium tumefaciens* strain GV3101/pMP90 that carries genes for resistance to gentamycin and rifampicin was used in this study (provided by Dr. S. Gelvin, Purdue University, West Lafayette, IN).

Two binary vectors, both derivatives of pBI121 (Clonetech, Palo Alto, CA) and designated pJSS-7 and pJSS-8, were developed. The pJSS-7 chimeric construct consisted of a 37 bp untranslated leader sequence of an alfalfa mosaic virus (AMV) RNA4 gene that was inserted between the β-glucuronidase (GUS) coding sequence and the CaMV 35S promoter. The AMV RNA4 leader sequence (Datla et al., 1993), provided by Dr. Susan Loesch-Fries (Purdue University, West Lafayette, IN), was added upstream of the *uidA* gene in pBI121. The forward oligonucleotide primer, corresponding to the 37 bp leader and the 24 bp from the *uidA* start codon (5′-cactctaga gtttttattt ttaattttct ttcaaatact tccatcatgg tccgtcctgt agaaacccca-3′ carrying an *Xba*I site), and the reverse primer (5′-cacgagctctttatttagttac taaatgcaata-3′ carrying an *Sst* I site) were used for amplification from pBI121. The amplified 1.9 kb fragment (AMV RNA4–GUS) was inserted into pBluescript KS- at *Xba*I and *Sst*I sites, and confirmed by sequencing. The 1.9 kb AMV RNA4–*uidA* fragment from pBluescript KS- was cut as a *BamH*I and *Sst*I fragment, and used to substitute the *uidA* gene in pBI121 at *BamH*I and *Sst*I sites. The pJSS-8 chimeric construct contained the *uidA* gene under the control of the E-8 fruit-ripening specific promoter and without any leader sequence. The pJSS-8 binary vector was a result of substituting the CaMV 35S promoter at *Hind*III and *BamH*I sites in pBI121 with a 2.2 kb E-8 promoter. Both vectors contained the *npt*II gene coding for kanamycin resistance, under the control of the *nos* promoter, as a selectable marker. The cloning vectors were then mobilized into *Agrobacterium* strain GV3101 via electroporation.

TABLE 1

COMPOSITION OF MEDIA FORMULATIONS USED FOR INDUCTION OF SHOOT ORGANOGENESIS IN TOMATO COTYLEDONS

Components of medium	Formulation of tomato regeneration (TR) medium							
	TR-1	TR-2	TR-3	TR-4	TR-5	TR-6	TR-7	TR-8
MS salts	1×	1×	1×	1×	1×	1×	1×	1×
B_5 vitamins	–	1×	1×	1×	1×	1×	1×	1×
Thiamine (mg l^{-1})	0.4	–	–	–	–	–	–	–
Inositol (mg l^{-1})	100	–	–	–	–	–	–	–
Glucose (mg l^{-1})	30	–	–	–	–	–	–	–
Sucrose (g l^{-1})	–	30	30	30	30	30	30	30
MES (mg l^{-1})	500	–	700	700	700	700	700	700
Zeatin (μ*M*)	2.28	–	2.28	–	–	2.28	–	–
Zeatin riboside (μ*M*)	–	5.69	–	–	–	–	–	–
IAA (μ*M*)	2.85	0.57	2.85	2.85	0.57	2.85	2.85	2.85
BA (μ*M*)	–	–	–	2.22	8.88	–	2.22	2.22
TDZ (μ*M*)	–	–	–	–	–	2.27	2.27	4.54
Agar (g l^{-1})	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0
pH	5.8	5.8	5.7	5.7	5.7	5.7	5.7	5.7

TR-1 and TR-2 correspond to media used by Hamza and Chupeau (1993) and Van Roeckel et al. (1993), respectively.

1× corresponds to full-strength MS salts (Murashige and Skoog, 1962) or full-strength B_5 vitamins (Gamborg et al., 1968).

Agrobacterium cells were grown overnight at 28°C in a YEP medium (10 g l^{-1} Bacto-peptone, 10 g l^{-1} Bacto-east, 10 g l^{-1} NaCl, pH 7.0) containing 100 mg l^{-1} of kanamycin, 40 mg l^{-1} rifampicin, and 50 mg l^{-1} gentamycin. Freshly grown cultures were used for transformation experiments.

Transformation, regeneration, and recovery of transgenic plants. Cotyledons were excised from *in vitro*-germinated 8-d-old seedlings of 'Swifty Belle'. These were placed in a suspension of an overnight-grown culture of *Agrobacterium* diluted with a liquid medium (designated TR-C) consisting of MS salts and B_5 vitamins. The pH was adjusted to 5.7 prior to autoclaving, while the OD_{600} of bacterial cells after dilution was adjusted to 0.01–0.1. Following a 30 min incubation period in the dark, cotyledons were blotted dry onto a filter paper, and placed on a fresh TR-C medium solidified with 10 g l^{-1} agar for 48 h under 16 h photoperiod (provided by 20 W cool-white fluorescent tubes yielding a light intensity of 30 μmol m^{-2} s^{-1}) and 25°C. Following cocultivation, cotyledons were washed twice in a liquid TR-C medium containing 500 mg l^{-1} carbenicillin, blotted dry, and placed on a selection medium. The selection medium consisted of the TR-8 regeneration medium (Table 1) solidified with 7 g l^{-1} agar, and containing 100 mg l^{-1} kanamycin and 500 mg l^{-1} carbenicillin. Explants were cultured for 5–6 wk under the same environmental conditions described above during which they were subcultured once (at 3 wk) to a fresh medium. Organogenic callus with shoot primordia was then transferred to a shoot proliferation medium (consisting of MS salts, B_5 vitamins, 4.56 μ*M* zeatin, 0.57 μ*M* indole-3-acetic acid (IAA), and solidified with 7 g l^{-1} agar; pH of medium adjusted to 5.7) containing 100 mg l^{-1} kanamycin and 500 mg l^{-1} carbenicillin. Shoots of 2–3 cm in length were excised, and placed on a rooting (TR-R) medium (MS salts, B_5 vitamins, 30 g l^{-1} sucrose, 7 g l^{-1} agar, and pH adjusted to 5.7) containing 100 mg l^{-1} kanamycin for secondary selection. Plantlets with developed roots were acclimatized, and transferred to the greenhouse for further growth and fruiting.

GUS assay. Histochemical analysis of GUS activity in leaves and fruits of transformed tomato plants using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Glu) as a substrate was carried out as described by Jefferson (1987). Following tissue-staining with X-Glu overnight at 37°C, chlorophyll from leaves and pigments from fruits were removed by soaking these tissues in a mixture of 70% ethanol and 10% commercial bleach Clorox® for 4–6 h. The ethanol–bleach mixture was replaced three or four times.

Fluorometric assay for GUS activity in crude tissue extracts from mature leaves and fruits (at the red-stage of ripening) was performed according to Jefferson (1987) using 4-methylumbelliferyl-β-D-glucuronide dihydrate (MUG) as a substrate. During leaf sampling, only mature leaves were collected as GUS activity was found to be consistently higher in mature than in young leaves (Schnurr and Guerra, 2000). A fluorometer (Hoefer DyNA Quant 2000, Amersham-Pharmacia Biotech, Arlington Heights, IL) was used for conducting fluorometric GUS assays. Total protein was measured using the Bradford method (Bio-Rad Protein Assay, Bio-Rad, Hercules, CA).

Southern blot. Genomic DNA was isolated from young leaves of putative transformed plants grown in the greenhouse using DNeasy Plant Minikit (Qiagen, cat. no. 69104, Valencia, CA). Extracted DNA was quantified and 10 μg was randomly digested overnight with *Hind*III, and electrophoresed in a 0.9% agarose gel. DNA was transferred to a nylon membrane (Hybond N+, Amersham-Pharmacia Biotech) using the capillary blotting technique. A 1.87 kb fragment of the *uidA* gene from pBI121 was cut using *Xba*I and *Sst*I restriction enzymes, extracted from the gel, labeled with ^{32}P, and used as a probe for Southern hybridization. Blotting, labeling, hybridization, and washing were carried out according to Sambrook et al. (1989). A λ-DNA restricted with *Hind*III was used as a molecular weight marker.

Segregation of T_1 plants. Seeds of T_0 plants (confirmed with Southern blotting and GUS assay) transformed with pJSS-7 and pJSS-8 were collected, stored in a cold chamber for 3 mo., and then germinated in flats in the greenhouse. T_1 plants were allowed to grow in 15-cm plastic pots containing a 1:1:1 (soil:peat:sand) mixture. After 4 wk of growth, leaf, stem and flower tissues were collected, and histochemical staining for GUS activity was performed. Most plants with pJSS-8 were lost due to lack of good watering practices, thus segregation for the *uidA* gene was determined only in plants with pJSS-7, and a chi-square analysis was conducted using the SAS statistical analysis package.

Results and Discussion

Effect of culture media on shoot organogenesis. Previous studies demonstrated that 8–10-d-old cotyledons of tomato were superior to other sources of explants, including hypocotyls, stems and leaves for promoting shoot organogenesis in tomato (Hamza and Chupeau, 1993; Van Roekel et al., 1993; Ling et al., 1998). Following a preliminary study where we compared shoot induction frequency using 8-, 9-, and 10-d-old cotyledons of 'Swifty Belle', we found that 8-d-old cotyledons showed a higher shoot induction frequency (data not shown) than either 9- or 10-d-old cotyledons, and therefore this age explant was used in all experiments conducted in this study.

As it was important to optimize the regeneration frequency of tomato to increase the likelihood of recovery of transformants, we evaluated several media formulations (Table 1) for their ability to induce shoot organogenesis in cotyledons of 'Swifty Belle'. The different formulations were designed based on a general survey of the most likely growth regulators and their concentrations that have been reported to promote shoot organogenesis from cotyledonary tissues of tomato. Since all media formulations induced shoot organogenesis in all cotyledon explants, the evaluation of induction of shoot organogenesis was based on number of shoots per explant. Among the eight formulations tested, the TR-8 medium, which contained 2.22 μM benzyladenine (BA) and 4.54 μM thidiazuron (TDZ) along with 2.85 μM IAA, induced the highest mean shoot number per explant, and was significantly different from all other formulations (Fig. 1). The presence of either BA or TDZ in the culture medium has long been reported to promote shoot organogenesis in a large number of plant species, but in some studies the presence of both growth regulators together enhanced the frequency of regeneration (Huetteman and Preece, 1993). In this study, combining both BA and TDZ in the culture medium resulted in a significant increase in mean number of shoots per explant, and by elevating the concentration of TDZ from 2.27 μM (TR-7) to 4.54 μM (TR-8), this further enhanced the frequency of shoot organogenesis in tomato explants (Fig. 1). This finding suggested presence of these two cytokinins in the culture medium had an invigorating effect on cell differentiation in tomato cotyledons.

Maintaining shoot regenerants on a medium containing TDZ for periods longer than 5–6 wk resulted in the recovery of short and compact shoots (<1 cm in length). Those shoots exhibiting this particular 'bushy' phenotype did not elongate any further in the presence of TDZ. Similar inhibitory effects of TDZ on shoot elongation have been described previously (Huetteman and Preece, 1993). To overcome this problem, shoot primordia and short shoots (3–5 mm in length) were excised from cotyledonary explants and transferred onto a shoot proliferation medium containing 4.56 μM zeatin and 0.57 μM IAA to promote proliferation and elongation of regenerated shoots.

Transformation and selection. Cotyledon explants were wounded and infected with *Agrobacterium* by submerging them into a bacterial suspension followed by cocultivation for 48 h at 25°C in the dark. Longer exposure of explants to *Agrobacterium*, for 72 h, did not improve transformation frequency, but resulted in 100% *Agrobacterium* regrowth after this cocultivation period and made it more difficult to eliminate *Agrobacterium* (data not shown). Following incubation on a selection medium, callus was formed on

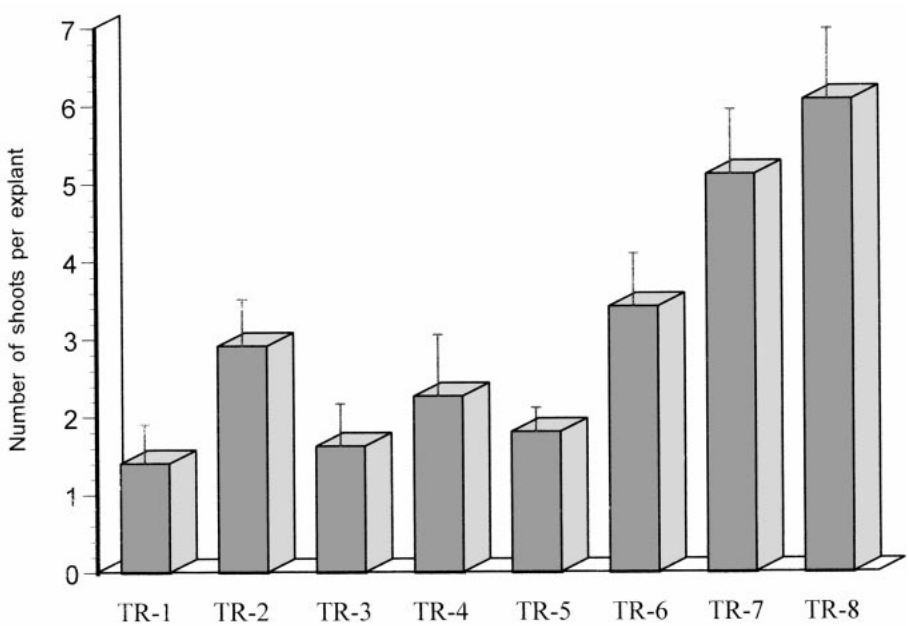

Fig. 1. Effect of different culture media formulations on shoot organogenesis of cotyledon explants of tomato cv. Swifty Belle.

TABLE 2

TOMATO TRANSFORMATION WITH *AGROBACTERIUM TUMEFACIENS* CARRYING pJSS-7 AND pJSS-8 CONSTRUCTS[a]

	pJSS-7	pJSS-8
No. of cotyledons per transformation	300	450
No. of shoots selected on kanamycin	49	52
No. of putative transformants (selected and rooted on kanamycin)	39	49
Transformation efficiency (%)[b]	13	10.9

[a] The construct pJSS-7 consists of the *uidA* reporter gene driven by the CaMV35S/AMV promoter; while the construct pJSS-8 consists of the *uidA* reporter gene driven by the E-8 fruit-specific promoter. This experiment was repeated once over time.

[b] Transformation efficiency was calculated as: No. of putative transformants/No. of cotyledons.

explants after 2 wk of culture. Two types of callus were observed, a brown-colored callus which was non-regenerable, and a white-colored callus which turned greenish within a few days, and was organogenic. This organogenic callus produced shoots after about 8–10 d. It was important to subculture the callus onto a fresh selection medium at least once to promote faster shoot formation, and to prevent *Agrobacterium* regrowth.

In previous reports (Joao and Brown, 1993; Hamza and Chupeau, 1993), 100 mg l^{-1} kanamycin was used for selection of putative tomato transformants. This same concentration of kanamycin was used in our selection medium as well. However, in this study, a secondary selection step was used whereby kanamycin at 100 mg l^{-1} was also added to the rooting medium to discriminate between non-transformed shoots (escapes) that did not express kanamycin resistance from putative transformed shoots that showed resistance to kanamycin.

Of putative transformed shoots, almost 80% of those transformed with the pJSS-7 construct and 94% of those transformed with the pJSS-8 construct were successfully rooted (Table 2). All rooted plants were transferred to soil, acclimatized, transferred to the greenhouse, and allowed to grow until fruiting. From each transformation experiment, 10–12 randomly selected putative transgenic plants were analyzed for the presence of the chimeric *uidA* gene using Southern blotting (Fig. 2). Eight out of 12 (transformed with pJSS-7) and seven out of 10 (transformed with pJSS-8) plants were confirmed to be transgenic. Seven transgenic plants from each group (transformed either with pJSS-7 or pJSS-8) were used for *uidA* gene expression studies.

Southern blot hybridization has revealed multiple insertion copy numbers in most genomic DNA of tomato plants transformed with either pJSS-7 or pJSS-8 constructs (Fig. 2). Approximately one to five copies of the *uidA* gene, ranging between 3.0 and 13.0 kb in size, are observed in these transgenic plants (Fig. 2). *Agrobacterium*-mediated transformation generally yields more stable integration of foreign DNA into a plant genome in comparison to direct gene transfer techniques (Pawlowski and Somers, 1996). However, some re-arrangements within the insert may occur following transformation as reported previously for this transformation scheme (Krasnyanski et al., 1999). In this study, we did not observe this phenomenon. All band sizes (for plants transformed with pJSS-7) are larger than 2.7 kb, which is the intact size of the CaMV 35S/AMV–*uidA* fusion gene, or 4.0 kb (for plants transformed with pJSS-8), which is the size of the E-8–*uidA* fusion gene.

Effect of promoters on GUS expression. Based on the histochemical GUS assays, GUS activity was detected in leaves, stems, flowers, and roots of transgenic plants, where the *uidA* gene was driven by CaMV 35S/AMV. No GUS staining was observed in untransformed plants. Similarly, no GUS staining was observed in any of the vegetative and floral tissues of transgenic plants carrying the *uidA* gene driven by the E-8 promoter. This latter finding is as expected as E-8 is a fruit-specific promoter and does not allow expression of a gene in any of the vegetative or floral tissues, but only in ripening fruit (Good et al., 1994).

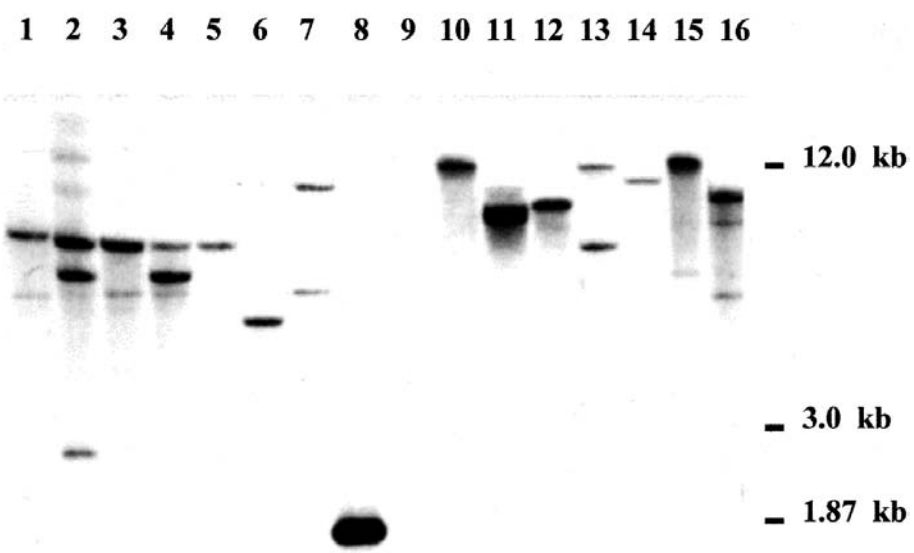

FIG. 2. Southern-blot hybridization of tomato cv. Swifty Bell plants transformed with the *uidA* gene driven by two different promoters. *Lanes 1–7*, tomato plants transformed with pJSS-7 (*uidA* gene under CaMV 35S/AMV promoter); *lane 8*, positive control (*uidA* gene); *lane 9*, negative control (untransformed tomato); *lanes 10–16*, tomato plants transformed with pJSS-8 (*uidA* gene under E-8 fruit-specific promoter).

In an attempt to visualize *uidA* gene expression in fruit tissues of both groups of transgenic plants, fruits at different stages of ripening, including mature-green, pink, and red (1975 USDA Standards for Grades of Fresh Tomatoes) were collected and used for X-Glu staining. After placing sliced fruit in a staining buffer overnight at 37°C, we found that all fruits including controls (collected from non-transgenic plants grown in the greenhouse and from cherry tomatoes purchased from a grocery store) developed faint and sporadic blue stains along the seeds and fleshy (including vascular) tissues, which were darker in color in ripened fruit. This GUS staining in control fruits has been attributed to the likely presence of endogenous GUS-like activity of unknown origin in the tomato fruit (Jelesko et al., 1999). Kosugi et al. (1990) proposed adding 20% methanol to the staining solution to overcome this problem. Another proposed solution involved maintaining the staining buffer at pH 7.0, as it has been known that the endogenous GUS enzyme was mostly active at pH 4–5, and thus by elevating the pH level of the staining buffer, this would suppress the endogenous activity. When the pH was measured in untransformed tomato fruit (juice squeezed from the flesh) at different stages of development, including green, pink, red, and red soft, the following pH readings were recorded: 3.72, 3.76, 5.09, and 5.20, respectively. When the staining buffer (with pH adjusted to 7.01) was added to thin cross-section slices of fruit, and samples were incubated overnight at 37°C, the pH values for all four samples changed to 5.08, 5.10, 5.09, and 5.20. At this point, tissues of all samples showed dark blue staining. To stabilize the pH of the staining buffer, 2-[*N*-morpholino]ethanesulfonic acid (MES) at concentrations of 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 mg l^{-1} was added to the substrate solution. It was found that 2 mg l^{-1} MES was optimal in stabilizing the final pH of the staining buffer (after samples were mixed), and maintaining it at 6.0. As a result, no dark blue color staining was observed in all tissues of the fruit, except for minimal presence of a barely visible light blue color along the vascular and seed tissues. Although this indicated that endogenous GUS-like activity was still present in untransformed fruit, it was easy to distinguish blue staining resulting from this endogenous activity from that of the *uidA* transgene expression in transgenic fruit at all stages of development.

To quantify *uidA* expression levels in transgenic plants, GUS activity was measured using fluorometric analysis. When fruit tissues of transgenic plants containing the *uidA* gene driven by the E-8 promoter were analyzed for GUS activity (Fig. 3a), varying levels of GUS activities were observed among the different plants, with an average value of 360 nmol MU (mg protein)$^{-1}$ min^{-1}. Similarly, when fruit tissues of transgenic plants analyzed for GUS activity containing the *uidA* gene driven by the CaMV 35S/AMV promoter, varying levels of GUS activities were observed among the seven different plants analyzed, however, the average GUS value for these plants was 943 nmol MU (mg protein)$^{-1}$ min^{-1}. Thus, on average, the GUS activity was 2.6-fold higher in fruits expressing the *uidA* gene driven by the CaMV 35S/AMV than by the E-8 promoter. This suggested that the CaMV 35S/AMV promoter was stronger than the E-8 promoter. In an earlier report, we compared the effectiveness of the CaMV 35S promoter with and without the AMV leader sequence on transient expression of an antigenic protein in apple protoplasts, and found that the insertion of the 5′-untranslated leader from alfalfa mosaic virus, AMV RNA4, between the CaMV 35S promoter and the antigenic gene increased viral protein expression by 5.5-fold (Sandhu et al., 1999).

No GUS activity was detected in leaf tissues of both E-8 transgenic and control plants tested; while varying levels of GUS activity were detected in leaf tissues of CaMV 35S/AMV transgenic plants (Fig. 3b). This observed difference in tissue specificity might be attributed to post-transcriptional regulation of GUS expression, as has been previously reported (D'Aoust et al., 1999). Therefore, whether we were dealing with stable or transient expression of a reporter or any other useful gene, expression levels of the gene of interest were significantly enhanced when the untranslated leader sequence of AMV RNA4 was added to the chimeric gene construct. This finding also confirmed previous reports (Warkentin et al., 1992; Charest et al., 1993; Datla et al., 1993; Haggman and Aronen, 1998). In this study, over 60% of transgenic tomato plants confirmed with Southern blot hybridization had multiple insertion copies of the *uidA* gene (Fig. 2). However, there was no correlation between gene copy number and level of GUS activity in these transgenic plants.

When levels of the GUS activity in fruit and leaf tissues were compared within the same plants transformed with pJSS-7 carrying the *uidA* gene driven by the CaMV 35S/AMV promoter, it was found that fruits had much higher levels (an average of 6.7-fold) of GUS activity than that found in leaf tissues (Fig. 3a, b). This might be attributed to either the developmental or physiological status of leaves during the reproductive phase of plant growth as leaves were analyzed during fruit set when old and fully-mature leaves were predominant. It has been reported that the highest GUS activity was usually observed in young rather than in mature leaves, and this might be correlated to accumulation of polyphenolic compounds which inhibit GUS activity in mature leaves (Serres et al., 1997). The level of GUS activity in leaf tissue of plants with pJSS-8 (the *uidA* gene under the E-8 promoter) or control plants was negligible, and similar to that of the background (Fig. 3b). This fluorometric analysis clearly showed that the E-8 fruit-ripening-specific promoter did not allow expression of the *uidA* gene in leaves, but only in fruits as has been previously reported (Good et al., 1994).

Segregation of T1 plants for the uidA gene. When T_1 plants, grown from T_0 transgenic plants carrying the pJSS-7 chimeric construct (the *uidA* gene driven by CaMV 35S/AMV), were evaluated for presence of the transgene, a 3:1 segregation ratio was observed among these plants suggesting a simple Mendelian inheritance of the the *uidA* transgene (Table 3). Expression of the *uidA* gene driven by the CaMV 35S/AMV was found in both leaf and fruit tissues, and its expression levels in T_1 plants carrying the transgene were similar to those found in T_0 plants (100–200 nmol MU (mg protein)$^{-1}$ min^{-1}).

In a random sampling, GUS activities in T_1 plants carrying the *uidA* gene driven by the CaMV 35S/AMV were almost 2–3-fold higher than those T_1 plants containing the *uidA* gene driven by the E-8 promoter.

Conclusions

This study found that combining both TDZ and BA in the tissue culture medium enhanced shoot organogenesis in tomato cotyledons. In addition, both a modified CaMV 35S promoter and a fruit-specific promoter successfully promoted *uidA* gene expression in tomato plants. However, the E-8 fruit-ripening-specific promoter clearly demonstrated its tissue specificity by expression of the *uidA* gene only within the fruit and not in leaf tissues. Insertion of the

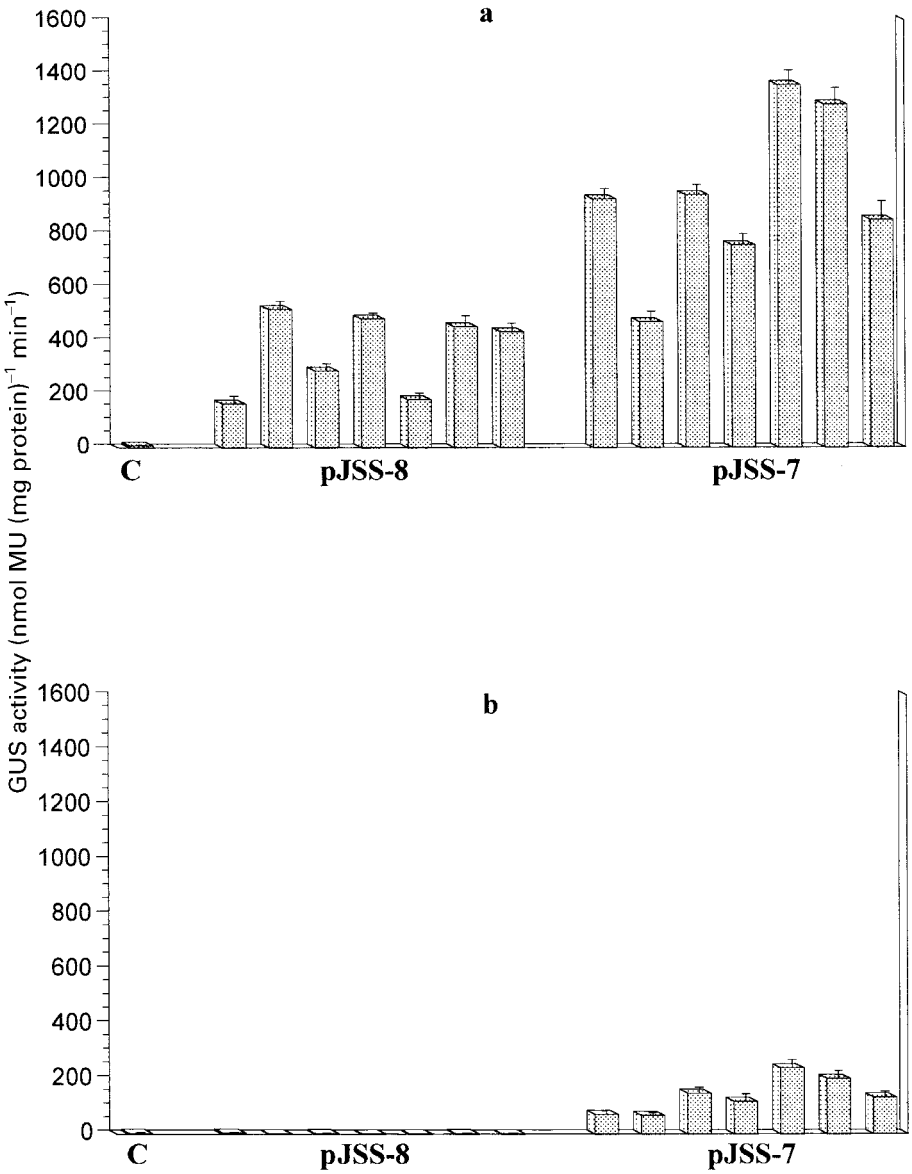

FIG. 3. GUS activity (fluorometric GUS assay) in fruit (a) and leaf (b) tissues of T_0 tomato plants transformed with pJSS-7 (*uidA* gene under CaMV 35S/AMV promoter) and pJSS-8 (*uidA* gene under E-8 promoter). C, negative control (untransformed tomato plants).

TABLE 3

SEGREGATION FOR THE *uidA* IN T_1 TOMATO SEEDLINGS TRANSFORMED WITH pJSS-7[a]

Transgenic line no.	No. of GUS-positive plants	No. of GUS-negative plants	Segregation ratio	Chi-square value	Significance level
79	14	4	3:1	0.073	0.787
41	17	5	3:1	0.060	0.807
98	28	11	3:1	0.213	0.644
38	8	3	3:1	0.030	0.862
78	17	6	3:1	0.014	0.906
44	19	8	3:1	0.026	0.872
43	24	7	3:1	0.032	0.858

[a] The construct pJSS-7 consists of the *uidA* reporter gene driven by the CaMV 35S/AMV promoter.

5′-untranslated leader of AMV between the CaMV 35S promoter and the *uidA* gene resulted in higher levels of GUS activity in tomato fruits in both T_0 and in segregating T_1 plants carrying the *uidA* gene.

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