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A COMBINATION OF OVERGROWTH-CONTROL ANTIBIOTICS IMPROVES *AGROBACTERIUM TUMEFACIENS*-MEDIATED TRANSFORMATION EFFICIENCY FOR CULTIVATED TOMATO (*L. ESCULENTUM*)

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Summary

The transformation efficiency of cultivated tomato (*Lycopersicon esculentum* cv. UC82) using *Agrobacterium tumefaciens* was improved from 14% in a previous report to 25% in the present study. Several variables potentially involved in the improvement of transformation efficiency were evaluated, including enhancements in the regeneration system, antibiotics used for *Agrobacterium*-overgrowth control, and method of applying kanamycin for selection. The most important variable identified was the influence of overgrowth-control antibiotics on both the regeneration response and transformation efficiency. The best transformant recovery and *Agrobacterium*-overgrowth control was obtained using 250 mg l^{-1} claforan and 250 mg l^{-1} ticarcillin as the overgrowth-control antibiotics in the media. Selfed T_1 progeny plants showed Mendelian inheritance ratios in 77% of the independently transformed lines according to phenotype expression [β-glucuronidase (GUS) assay results], and confirmed by polymerase chain reaction amplification of the transgene in progeny.

Key words: claforan; ticarcillin; vancomycin; transgene inheritance test.

Introduction

Tomato is one of the better studied higher plants because it is an important crop species with several advantages for genetic, molecular and physiological studies (McCormick et al., 1986). Transformation is being used as a basic research tool in plant biology and a practical tool for cultivar improvement (Newbigen et al., 1995; Staskawicz et al., 1995; Herbers and Sonnewald, 1996). *Agrobacterium tumefaciens* provides a natural gene transfer mechanism that can be used to transfer specific DNA sequences into the genome of various plants (Gasser and Fraley, 1989; Birch, 1997). Transgenic plants of *Lycopersicon esculentum* 'UC82', a commercial processing cultivar, have been obtained using *A. tumefaciens*-mediated transformation and regeneration from both leaf and cotyledon explants (McCormick et al., 1986; Fillatti et al., 1987). Several studies have attempted to improve tomato transformation procedures (Horsch et al., 1985; McCormick et al., 1986; Fillatti et al., 1987; Davis et al., 1991; Hamza and Chupeau, 1993). Although Fillatti et al. (1987) reported high transformation frequencies for 'UC82' tomato (58–73%) based on kanamycin resistance assays, the results of Hamza and Chupeau (1993) called into question the reliability of estimating transformation events in this manner. Hamza and Chupeau (1993) re-evaluated the previously reported tomato transformation methods, and developed an improved culture media sequence including the use of feeder cells during explant preculture and cocultivation. These authors confirmed that high frequencies of putative transformation events were estimated by kanamycin resistance assays alone (55%), but that the actual recovery of confirmed transformed plants remained relatively low (14% for 'UC82B').

A transformation system using *A. tumefaciens* can be viewed as including two major stages: transformation of plant cells by the engineered *Agrobacterium* through cocultivation; and recovery of transformed plants from transformed cells through selection and regeneration (Chyi and Phillips, 1987). Cocultivation concerns the interaction between *Agrobacterium* and explant tissues. Many factors are involved in this interaction. Both cointegrated and binary vectors have been effective in tomato transformation (Horsch et al., 1985; McCormick et al., 1986). Preculture of explants prior to cocultivation has improved transformation in some species, e.g. *Arabidopsis* (Sangwan et al., 1991). Phytohormones or growth regulators included in preculture media stimulate the production of competent cells through more active cell division (An, 1985; Valvekens et al., 1988).

Cellular transformation does not always lead to regeneration of transformed buds and shoots, since not all transformed cells are competent for regeneration (Dong and McHughen, 1991). Growth regulators used in regeneration media affect the formation of adventitious buds and shoots. Antibiotics which are commonly used to eliminate *A. tumefaciens* from plant tissue during transformation experiments have been shown to influence morphogenesis either positively or negatively (Eapen and George, 1990; Chang and Schmidt, 1991; Lin et al., 1995; Ling et al., 1998). Kanamycin, a widely used selection agent for plant transformation, strongly inhibited apple regeneration even when used at low doses (Yepes-Martinez and Aldwinckle, 1994).

Many of these studies demonstrated recovery of primary

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transformants without demonstration of transgene inheritance. Although progeny tests were conducted by McCormick et al. (1986) and Fillatti et al. (1987) for tomato, the sample sizes were insufficient for statistical analysis to evaluate transformation qualities.

The aims of this study were to attempt to further improve the transformation efficiency of cultivated tomato (*L. esculentum* 'UC82') through manipulation of the cocultivation, selection, and regeneration procedures; and to evaluate transformant quality through a T_1 progeny test.

Materials and Methods

Standard regeneration of shoots. (1) Seed germination: *L. esculentum* cv. UC82 seeds were surface sterilized and germinated on BSG medium (modified 0.1-strength B5 medium; Phillips and Luteyn, 1983) at 28°C under 24 h light (15 μmol m^{-2} s^{-1}) for 8 d. (2) Preparation of tobacco cell feeder: Tobacco (cv. Xanthi) cell suspension (0.33 ml) was plated onto the surface of AB medium [MS basal medium (Murashige and Skoog, 1962) plus 3% sucrose, MW vitamins (Morel and Wetmore, 1951), 0.7% plant tissue culture agar, 5.4 μ*M* α-naphthaleneacetic acid (NAA) and 4.5 μ*M* 2,4-dichlorophenoxyacetic acid (2,4-D); Hamza and Chupeau, 1993]. A disc of sterile filter paper was placed on top of the tobacco cell feeder layer. (3) Initiation of shoot buds: 8-d-old (from time of culture on BSG medium) tomato cotyledons were excised from aseptically germinated seedlings. Both ends of the cotyledons were trimmed to generate rectangular tissues, and the rectangular tissues were each cut into two sections, resulting in wounded cotyledon explants. These tomato explants were placed on top of the filter paper covering the tobacco cell feeder on AB medium for 3 d (1 d preculture, 2 d cocultivation), then explants were transferred onto AZ medium [MS basal medium plus 500 mg l^{-1} 2-*N*-morpholinoethane sulfonic acid (MES), 2.9 μ*M* indole-3-acetic acid (IAA), 2.3 μ*M* zeatin, 30 g l^{-1} glucose, 3.2 g l^{-1} Phytogel; Hamza and Chupeau, 1993] for 2 wk to initiate shoot buds. (4) Shoot elongation: After shoot buds developed on the wounded sites, explants were transferred onto MS119 medium [MS basal medium plus 29.4 μ*M* $AgNO_3$, 8.8 μ*M* 6-benzylaminopurine (BA), 6.1 μ*M* gibberellic acid (GA_3), 30 g l^{-1} glucose, 3.2 g l^{-1} Phytogel; Hyde and Phillips, 1996] to develop shoots.

Regeneration variations tested. (1) Initiation of shoot buds: Wounded 8-d-old cotyledon explants were cultured onto: (a) procedure 1: AB and AZ media as described above, (b) procedure 2: MS101 medium (MS basal medium plus 2.9 μ*M* IAA, 8.8 μ*M* BA, 30 g l^{-1} glucose, 3.2 g l^{-1} Phytogel; Hyde and Phillips, 1996) with tobacco cell feeder for 3 d, and then MS101 medium lacking tobacco cell feeder for 2 wk, and (c) procedure 3: MS85 medium (MS basal medium plus 4.4 μ*M* BA, 30 g l^{-1} glucose, 3.2 g l^{-1} Phytogel; Chyi and Phillips, 1987) with tobacco cell feeder for 3 d, and then MS85 medium lacking tobacco cell feeder for 2 wk. (2) Shoot elongation: Cotyledon explants with shoot buds initiated from AZ, MS101 or MS85 media were either: (a) subcultured onto fresh plates of the original media (AZ, MS101 or MS85, respectively) for 2 mo., or (b) transferred onto MS119 media for 2 mo. For both methods, subculture to fresh media was performed every 2 wk. At least 40 cotyledon explants were tested per replication with at least three replications for each treatment.

Transformation. (1) *Agrobacterium* strain and plasmid: C58C1 (pGV 2260) *Rif* resistant *A. tumefaciens* strain was used. The pBI121 plasmid was used as the binary vector (Fig. 1). (2) *A. tumefaciens* culture: *Agrobacterium* stocks were grown on semi-solid selection medium [LB medium (Sambrook et al., 1989) plus 100 mg l^{-1} kanamycin, 40 mg l^{-1} rifampicin, 1% agar]. A single colony was selected and grown in 10 ml liquid selection medium (LB medium plus 75 mg l^{-1} kanamycin, 20 mg l^{-1} rifampicin) overnight at 29°C on a rotary shaker. (3) Inoculation: Overnight-grown *Agrobacterium* liquid culture (1.5 ml) was placed into a microcentrifuge tube, centrifuged at 12 000 rpm for 1 min, and resuspended in 1.5 ml antibiotic-free LB liquid medium. Explants were immersed in *Agrobacterium* (with antibiotic-free medium) for 10 min inoculation after 1-d preculture on AB medium with tobacco cell feeder. (4) Cocultivation: After inoculation, explants were blotted on a sterile filter paper to remove excess *Agrobacterium*, and then placed onto AB medium with tobacco cell feeder for 2 d cocultivation at 28°C in the dark. (5) Washing cocultivated explants: After 2 d cocultivation, explants were immersed in 1000 mg l^{-1} claforan solution for 10 min to remove *Agrobacterium* on the explant surface, and rinsed 3× with sterile water. (6) Regeneration of transformants: After washing, explants were placed on a sterile filter paper to blot dry. Explants were transferred onto AZ medium containing 50 mg l^{-1} kanamycin + 250 mg l^{-1} claforan + 250 mg l^{-1} ticarcillin for bud initiation (2 wk), and then transferred onto MS119 medium containing 150 mg l^{-1} kanamycin + 250 mg l^{-1} claforan + 250 mg l^{-1} ticarcillin for shoot elongation (2 mo. total with subculture every 2 wk to fresh media); except where stated otherwise.

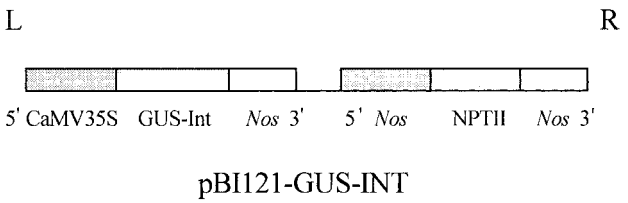

Fig. 1. Sketch of the gene structure for the pBI121-GUS-INT insert contained in the pBI121 binary plasmid used for tomato transformation (not to scale). L is the left border of T-DNA and R is the right border of T-DNA. Darkest boxes represent promoters, darker boxes represent coding regions, and light boxes represent terminators. GUS-INT coding sequence including an intron was used as the reporter gene and NPTII coding sequence was used as the selectable gene.

Overgrowth-control antibiotics. Antibiotics were tested for effectiveness at controlling *Agrobacterium* overgrowth, and influences on explant regeneration and transformation efficiency (the proportion of treated explants giving rise to at least one β-glucuronidase (GUS) positive shoot, expressed as a percentage): (1) either 250 or 500 mg l^{-1} vancomycin; (2) either 250 or 500 mg l^{-1} ticarcillin; (3) either 250 or 500 mg l^{-1} claforan; (4) 250 mg l^{-1} vancomycin plus 250 mg l^{-1} ticarcillin; (5) 250 mg l^{-1} ticarcillin plus 250 mg l^{-1} claforan; (6) 250 mg l^{-1} vancomycin plus 250 mg l^{-1} claforan; (7) 250 mg l^{-1} ticarcillin plus 250 mg l^{-1} vancomycin plus 250 mg l^{-1} claforan. These antibiotics were added into AZ medium for bud initiation and MS119 medium for shoot elongation.

Kanamycin selection. The concentration and timing of kanamycin used for selection of transformed shoots were tested: (1) AZ and MS119 media both with either 50, 100, 150 or 200 mg l^{-1} kanamycin; (2) AZ medium with 50 mg l^{-1} kanamycin, followed by MS119 media with 50, 100, 150 or 200 mg l^{-1} kanamycin.

Transformant rooting and plantlet maturity in greenhouse. Primary transformed shoots (T_0 generation) were transferred onto MS0 medium (no growth regulators) plus 50 mg l^{-1} kanamycin (used to enforce selection against escapes) in Magenta GA-7 boxes to develop roots. Once prolific root systems were established, plantlets were gently removed from the boxes and semi-solid medium was rinsed off from roots. Plants were placed in small pots and roots were placed straight down in the soil. Roots were covered with dry, fine soil and watered. Pots with plants were covered with a plastic bag to maintain high relative humidity. The plastic bag was opened for 1 h daily to add fresh air, and plants were lightly watered. About 5 d later, the plastic bag was removed and plants were grown under greenhouse conditions. Plants later were transferred into larger pots for maturation and seed collection of the self-pollinated T_1 generation.

Progeny test. T_1 generation seeds from each of 44 independent transformant lines were germinated on BSG media for 10 d. Shoot tips were excised and transferred onto MS0 medium for 10 d for shoot growth. Preliminary experiments indicated that kanamycin resistance assays were not as reliable as the GUS assay in distinguishing transformed from nontransformed seedlings. Thus, GUS assays were performed on true leaves of each seedling to analyze segregation of transgenic (GUS positive) and nontransgenic (GUS negative) progeny. Ten fruits were collected from each independent transformant and 10 T_1 seeds were tested from each fruit (for a total of 100 progeny tested per line).

GUS assay. GUS activity was detected histochemically among leaf samples of regenerated shoots, and true leaves of T_1 seedlings. Leaf pieces were excised and incubated with 10–20 μl GUS solution (94% phosphate buffer, 1% Triton/ethanol, 5% X-Gluc stock) in a well of a 96-well plate. The plate was sealed to keep tissues moist, incubated at 37°C for at least 3 h,

and tissues were examined under a dissection scope. The presence of blue stains on the cut portion of the leaf was scored as positive GUS activity. If the presence of chlorophyll obscured the detection of blue stains, then the solution was removed from the wells with the aid of a Pasteur pipette, and the tissues were washed with 95% ethanol overnight at room temperature (Jefferson, 1987). Three pieces were taken from each of three individual leaves of each plant assayed.

Polymerase chain reaction (PCR) analysis. T_1 progeny plants from 11 randomly selected independent transformant lines and two untransformed F_1 seedlings were collected and DNA was isolated from each test plant. All DNA isolation methods and chemicals were purchased from *QIAGEN* and described in *DNeasy Plant Mini Handbook* (Qiagen, Valencia, CA). *DyNA Quant* 200 (Amersham Pharmacia Biotech, Piscataway, NJ) was used for DNA quantification following extraction. Assay solution (2 ml consisting of water 90%, 10× THE buffer 10%, and H33258 stock solution 0.01%) was placed in a cuvette and 2 μl of sample DNA was added and mixed in the cuvette. The fluorometer was used to read the sample (the concentration in the cuvette is the reading in ng ml^{-1}). The reaction system contained 33 μl H_2O, 5 μl buffer, 2 μl dNTP (100 m*M*), 3 μl $MgCl_2$, 1 μl primer pair (forward-primer + reverse-primer), 1 μl *Taq* polymerase and 10 μl DNA sample. The sequence for GUS forward-primer is 5′CCATTTGAAGCCGCTGTCAC3′. The sequence for GUS reverse-primer is 5′TGGCGGTAACAAGAAAGGG3′. After mixing, the reaction was amplified. Amplifications were performed for 35 cycles with each cycle comprised of 1 min 95°C, 1 min 52°C and 2 min 70°C by using a DNA Thermal Cycler. Powdered agarose at 1% + 1 × TAE [48.4 g l^{-1} Tris base + 1.14 g l^{-1} glacial acetic acid, 2 ml 0.5 *M* EDTA (pH 8.0)] were used for the electrophoresis gel, and 1 × TAE was used for the electrophoresis buffer. A mixture of 90% sample DNA with 10% gel-loading buffer (50% glycerol, 0.13 *M* EDTA, 1% sodium dodecyl sulfate (SDS), ddH_2O, Bromophenol Blue) was loaded and 60 V current applied for the electrophoresis run. After electrophoresis, the gel was stained with ethidium bromide for 30 min. The gel was examined under ultraviolet light and photographed.

Statistical analysis. Analysis of variance and Duncan's multiple range test were used to compare treatment effects in the regeneration and transformation studies (Dowdy and Wearden, 1991). A χ^2 test was used to evaluate T_1 segregation ratios (Dowdy and Wearden, 1991).

Results and Discussion

Bud initiation and shoot elongation. Shoot regeneration efficiency often is positively correlated with transformation efficiency (the proportion of treated explants giving rise to at least one GUS positive shoot, expressed as a percentage). Variations of three regeneration procedures were compared for tomato in this study. The first procedure was based on Hamza and Chupeau (1993), where cotyledon explants were cultured on AB medium with tobacco cell feeder for 3 d to stimulate cell division at wounded sites, and then explants were transferred onto AZ medium to initiate shoot buds for 2 wk. After initiation of shoot buds, explants were either maintained on AZ medium as per Hamza and Chupeau (1993) or transferred onto MS119 medium (as per Hyde and Phillips, 1996) for shoot elongation. The second procedure was based on a pepper system developed in our laboratory (Hyde and Phillips, 1996), where cotyledon explants were cultured on MS101 medium to initiate shoot buds for 2 wk. Afterwards, some explants were maintained on MS101 medium and others were transferred onto MS119 medium for shoot elongation. The third procedure was based on an alternate tomato system developed in our laboratory (Chyi and Phillips, 1987), where cotyledon explants were cultured on MS85 medium to initiate shoot buds for 2 wk, and then either maintained on MS85 medium or transferred onto MS119 medium for shoot elongation.

Different responses for shoot bud initiation and shoot elongation were obtained using these varying procedures (data not shown). The highest frequency of shoot bud initiation was obtained using the Hamza and Chupeau (1993) procedure (AB → AZ media). The frequency of explants showing shoot bud induction on AB → AZ media (64.2%) was significantly higher than that on MS101 medium (48.4%), but statistically comparable to that on MS85 medium (59.4%). The number of shoot buds per explant obtained using AZ medium (9.4) was significantly higher than those on MS101 (3.8) and MS85 media (2.5). The highest frequency of shoot elongation was obtained in the first procedure, using MS119 medium after transfer from AZ medium (60% elongation, 2.3 shoots per explant). Although the use of MS119 following AZ was significantly better for shoot elongation than the continued use of AZ medium (30% elongation) as in the Hamza and Chupeau (1993) procedure, there were statistically comparable shoot elongation responses among the other regeneration procedure variations tested (42–55% elongation, 1.7–1.8 shoots per explant). The presence of 2,4-D in the AB medium, used for a 3-d preculture before the AZ medium in this procedure, might have stimulated more callus formation on explant wound sites, and therefore there may have been numerically more competent cells that could differentiate into shoot buds on AZ medium. Explants subsequently transferred to MS119 media showed less callus formation, perhaps allowing more shoot buds to elongate. The presence of GA in MS119 also may have promoted shoot elongation.

Antibiotics used for Agrobacterium*-overgrowth control.* Antibiotics used for controlling *Agrobacterium* overgrowth (250 or 500 mg l^{-1} claforan, ticarcillin, or vancomycin) were added into both AZ medium (bud initiation) for 2 wk and MS119 medium (shoot elongation) for 2 mo. (with 2-wk subcultures to fresh media). The regeneration response, overgrowth frequency at 30 and 60 d after cocultivation, and transformation efficiency were assessed.

Explants cultured with 250 or 500 mg l^{-1} vancomycin (Table 1) showed no significant difference in regeneration response with (68.8% and 87.5%, respectively) or without *Agrobacterium* treatment (76.7% and 85.4%, respectively). Vancomycin treatments showed low *Agrobacterium*-overgrowth frequencies at 30 d after inoculation, but both vancomycin levels permitted 100% *Agrobacterium*-overgrowth at 60 d after inoculation. Transformation efficiencies using 250 or 500 mg l^{-1} vancomycin were 16.7% and 18.0%, respectively.

Explants cultured on media with 250 or 500 mg l^{-1} ticarcillin showed no significant difference in regeneration response (96.7% and 93.3%, respectively) without *Agrobacterium* treatment (Table 1). In contrast, explants cultured on media with 250 mg l^{-1} ticarcillin showed significantly lower regeneration efficiency (36.7%) after *Agrobacterium* treatment than those cultured on 500 mg l^{-1} ticarcillin (65.0%), probably because *Agrobacterium* overgrowth damaged more potential shoot buds on the lower ticarcillin treatment. At 30 d after inoculation, the *Agrobacterium*-overgrowth frequency for 250 or 500 mg l^{-1} ticarcillin treatments were 78.3% and 57.4%, respectively, but at 60 d *Agrobacterium* overgrowth was 100% for both ticarcillin levels. A higher transformation efficiency was obtained using 500 mg l^{-1} ticarcillin (13.3%) than that obtained using 250 mg l^{-1} ticarcillin (6.7%), perhaps due to the rapid onset of *Agrobacterium* overgrowth on the lower ticarcillin treatment.

Explants cultured with 500 mg l^{-1} claforan showed significantly lower regeneration response (58.0%) than those cultured with

TABLE 1

REGENERATION AND TRANSFORMATION RESPONSES OF 'UC82' TOMATO COTYLEDON EXPLANTS USING DIFFERENT ANTIBIOTIC TREATMENTS FOR CONTROL OF *AGROBACTERIUM* OVERGROWTH AND AFTER SELECTION WITH KANAMYCIN FOR 10 WK

	Vancomycin		Ticarcillin		Claforan	
Concentrations of antibiotics (mg l^{-1})	250	500	250	500	250	500
No *Agrobacterium* treatment, regeneration frequency (%)	76.7 a	85.4 a	96.7 a	93.3 a	81.3 a	58.0 b
Agrobacterium treatment, regeneration frequency (%)	68.8 a	87.5 a	36.7 b	65.0 a	47.2 ab	35.9 b
Agrobacterium-overgrowth frequency (%) at 30 d	10.5	0.0	78.3	57.4	0.0	0.0
Agrobacterium-overgrowth frequency (%) at 60 d	100.0	100.0	100.0	100.0	0.0	0.0
Transformation efficiency, GUS positive shoots per 100 explants	16.7	18.0	6.7	13.3	7.0	10.0

Values within rows followed by the same letter are not statistically different at $\alpha = 0.05$ using the Duncan multiple range test. Kanamycin (50 mg l^{-1}) and overgrowth control antibiotics were added into AZ (2 wk) and MS119 media (8 wk total, with subculture every 2 wk to fresh media). Transformation efficiency is defined as the proportion of treated explants giving rise to at least one GUS positive shoot.

TABLE 2

REGENERATION AND TRANSFORMATION RESPONSES OF 'UC82' TOMATO COTYLEDON EXPLANTS USING DIFFERENT ANTIBIOTIC COMBINATIONS FOR CONTROL OF *AGROBACTERIUM* OVERGROWTH AND AFTER SELECTION WITH KANAMYCIN FOR 10 WK

Antibiotics used for overgrowth control (mg l^{-1})	250 ticarcillin +250 claforan	250 ticarcillin +250 vancomycin	250 vancomycin +250 claforan	250 ticarcillin +250 claforan +250 vancomycin
No *Agrobacterium*, regeneration frequency (%)	95.6 a	77.0 b	75.9 b	64.9 b
Agrobacterium treatment, regeneration frequency (%)	75.7 a	75.8 a	73.9 a	58.8 b
Agrobacterium-overgrowth frequency (%) at 60 days	0.0	15.7	0.0	0.0
Transformation efficiency, GUS positive shoots per 100 explants	24.8 a	15.3 ab	13.3 b	9.8 b

Values within rows followed by the same letter are not statistically different at $\alpha = 0.05$ using the Duncan multiple range test. Kanamycin (50 mg l^{-1}) and overgrowth-control antibiotics were added into AZ (2 wk) and MS119 media (8 wk total, with subculture every 2 wk to fresh media).

250 mg l^{-1} claforan (81.3%) without *Agrobacterium* treatment (Table 1), but regeneration responses were comparable with *Agrobacterium* treatment. Both claforan treatments showed complete *Agrobacterium*-overgrowth control 60 d post-inoculation, but low transformation efficiencies were obtained using 250 mg l^{-1} (7.0%) or 500 mg l^{-1} claforan (10.0%).

With respect to control (without *Agrobacterium* treatment) regeneration response, ticarcillin > vancomycin > claforan; but with *Agrobacterium* inoculation, regeneration frequencies were better using vancomycin (Table 1). β-Lactam antibiotics, such as penicillin and cephalosporins, are among the most commonly used antimicrobial agents. Ticarcillin, vancomycin and claforan (also called cefotaxime) belong to the β-lactam class of antibiotics. Some β-lactams, such as carbenicillin and penicillin G, have been shown to possess auxin-like structural features (Robert et al., 1989), and are broken down to physiologically active levels of the auxin phenylacetic acid in cultures (Holford and Newbury, 1992). The resulting stimulation of cell division due to the release of auxin-like compounds by these antibiotics has been shown to enhance callus formation and shoot regeneration, especially when using ticarcillin (Ling et al., 1998; Costa et al., 2000).

Agrobacterium-overgrowth frequencies were high using vancomycin and ticarcillin treatments, while the overgrowth frequency using claforan was zero at 60 d (Table 1). Transformation efficiencies were better using 250 or 500 mg l^{-1} vancomycin (16.7% and 18.%, respectively), compared to ticarcillin and claforan treatments. Thus, with respect to *Agrobacterium*-overgrowth control, claforan > vancomycin > ticarcillin; but with respect to transformation efficiency, vancomycin > ticarcillin > claforan.

Because the different individual antibiotics had varying influences on tomato regeneration, *Agrobacterium*-overgrowth control and transformation rates, we hypothesized that a combination of these antibiotics might prove effective for both regeneration and transformation of tomato (Table 2). Using the antibiotic combinations in AZ and MS119 media without *Agrobacterium* treatment, the regeneration frequency with ticarcillin + claforan (95.6%) was significantly higher than those with the other combinations tested, suggesting that the combination of ticarcillin + claforan could support tomato regeneration. Following *Agrobacterium* treatment, ticarcillin + claforan (75.7%), ticarcillin + vancomycin (75.8%) and vancomycin + claforan (73.9%) treatments were significantly better for regeneration than was ticarcillin + vancomycin + claforan (57.9%), suggesting the combination of ticarcillin + vancomycin + claforan inhibited regeneration. All combinations controlled *Agrobacterium* overgrowth completely up to 60 d after inoculation, except for the ticarcillin + vancomycin treatment (15.7%). Explants cultured on medium with ticarcillin + claforan showed the highest transformation efficiency (24.8%). Explants with ticarcillin + vancomycin (15.3%) and vancomycin + claforan (13.3%) showed moderate transformation frequencies, while ticarcillin + vancomycin + claforan (9.8%) supported the least transformation. Thus, regeneration response, *Agrobacterium*-overgrowth control and transformation efficiency were improved by using a combination of 250 mg l^{-1} ticarcillin which stimulated

TABLE 3

THE DISTRIBUTION OF GUS SEGREGATION RATIOS AMONG PROGENIES OF 44 INDEPENDENT TRANSFORMANT LINES OF 'UC82' TOMATO

	Segregation ratio					
	0	<3:1	3:1	<15:1	15:1	63:1
Apparent transgene copy number[a]	Null	1 locus chimeric	1 locus	2 loci chimeric	2 loci	3 loci
Frequency among independent transformed lines (%)	4.5	13.6	56.8	4.5	18.2	2.3

[a] Based on χ^2 analysis.

regeneration, and 250 mg l^{-1} claforan which controlled *Agrobacterium* overgrowth. Some previous reports indicated that combinations of antibiotics were effective in stimulating callus formation and shoot regeneration, and eliminating *Agrobacterium* (Chang and Schmidt, 1991; Ling et al., 1998).

Kanamycin used for transformant selection. Kanamycin was used to select transformed cells in AZ and MS119 media. Kanamycin application was tested in two ways. In the first group, constant concentrations of 50, 100, 150 or 200 mg l^{-1} kanamycin treatments were used both in AZ medium (bud initiation) for 2 wk and MS119 medium (shoot elongation) for 2 mo. (with 2-wk subcultures to fresh media). In the second group, 50 mg l^{-1} kanamycin was used in AZ medium for 2 wk followed by 50, 100, 150 or 200 mg l^{-1} kanamycin treatments in MS119 medium for 2 mo.

For the first group using constant selection pressure, regeneration responses decreased and transformation efficiencies increased as the kanamycin concentration was increased between 50 and

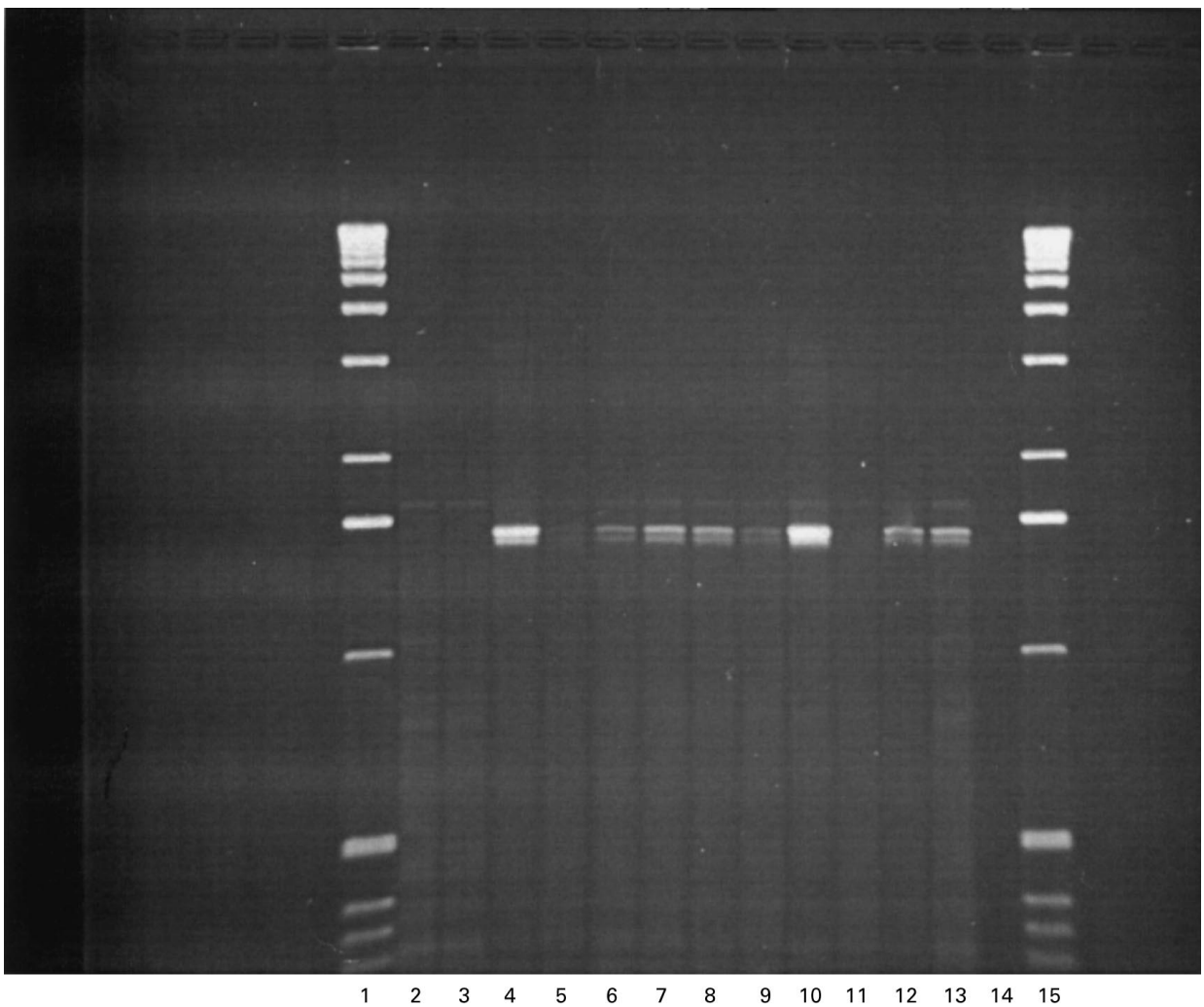

FIG. 2. PCR analysis for the presence of the GUS gene in T_1 seedling progenies derived from selfed primary transformant (T_0) 'UC82' tomato plants. Lanes 1, 15 = molecular markers; lanes 2, 3 = nontransformed F_1 control 'UC82' seedlings; lanes 4–14 = T_1 seedlings testing positive for GUS activity.

200 mg l^{-1} (data not shown). Kanamycin treatment at 50 mg l^{-1} resulted in high regeneration efficiency (57%) but low transformation efficiency (9%). In contrast, 200 mg l^{-1} kanamycin resulted in low regeneration efficiency (27%) but higher transformation efficiency (16%). The difference between the treatments for regeneration was significant but was not significant for transformation. These results suggest that regeneration was inhibited with increasing kanamycin concentration, and are consistent with a previous report in tomato (Ling et al., 1998). Similar results were obtained in apple (Yepes-Martinez and Aldwinckle, 1994) and *Solanum gilo* (Blay and Oakes, 1996).

For the second group of treatments in this study where selection pressure was low initially and then increased, the regeneration frequencies were not significantly different while transformation efficiencies were significantly different when the higher concentrations of kanamycin were used in comparison to the lowest concentration in MS119 medium (data not shown). Regeneration responses for all kanamycin (50–200 mg l^{-1}) treatments in MS119 medium were high (50–70%), and the best transformation efficiencies occurred when 50 mg l^{-1} kanamycin was used in AZ medium and 150 mg l^{-1} (25%) or 100 mg l^{-1} kanamycin (23%) was used in MS119 medium. These results suggest that tomato regeneration and selection procedures were optimal when 50 mg l^{-1} kanamycin was used for shoot bud initiation (AZ media) and higher kanamycin concentrations (e.g. 150 mg l^{-1}) were used for shoot elongation (MS119 media).

Inheritance test. Selfed T_1 seed progenies from putative transformed plants were tested for stability of transgene expression, copy number of transgene(s), and chimeric transformation as detected through inheritance patterns (Table 3). A total of 44 independently transformed lines were studied for GUS inheritance. According to the GUS assays, 77.3% of the seedling progenies from independent transformant lines were shown to fit typical Mendelian segregation ratios (56.8% showing 3:1 ratio indicating one transgene locus, 18.2% showing 15:1 ratio indicating two transgene loci, and 2.3% for 63:1 ratio indicating three transgene loci). Segregation data permit the determination of the number of independent T-DNA inserts present in the transformed clones (Katavic et al., 1994). The results also showed that 18.1% (13.6% for <3:1 and 4.5% for <15:1) of the transformant lines showed non-Mendelian segregation ratios (Table 3). Thus, the results from this study suggested that stable transformation with primarily single-copy insertion events was achieved, but some multiple-copy transformation and chimeric events were also obtained. Non-Mendelian segregation might be due to chimerism in the primary transformants containing both transformed and non-transformed cells (McHughen and Jordan, 1989). Progeny showing no GUS inheritance might be the result of gene silencing (Bruening, 1998), or perhaps these primary transformants were chimeric with no GUS transgene in the germ line cells. Both single- and multiple-copy insertion events in transgenic tomato were suggested in the progeny test conducted by McCormick et al. (1986).

PCR analysis was performed with 11 randomly selected T_1 seedlings (Fig. 2). Lanes 4–14 represent T_1 seedlings from GUS positive primary transformed plants, while lanes 2 and 3 represent F_1 seedlings from nontransformed control plants. The GUS gene used in our constructs was amplified and the size of this gene was estimated to be about 1.4 kb. All transformed seedlings showed 1.4 kb bands (lanes 4–14). Although the bands are difficult to see in lanes 5, 11 and 14, they were distinctly seen in the original gel. The characteristic 1.4 kb band was absent for both nontransformed control seedlings (lanes 2 and 3). We concluded that the 1.4 kb bands represented the amplified GUS genes in the progeny. In addition to the 1.4 kb bands in the PCR analysis, however, there was an additional 1.6 kb band in transformed seedlings. This 1.6 kb band might be the result of the 5′ primer used for GUS amplification also annealing at an unexpected location near the GUS gene. This interpretation is based on the fact that there were some bases in the 5′ primer that could have paired with similar sequences in the surrounding DNA of the GUS construct. Nevertheless, the PCR analysis provided additional evidence that GUS genes were integrated into the tomato genome, and that transgene transmission was stable in progeny.

Conclusions

The Hamza and Chupeau (1993) tomato regeneration procedure (AB → AZ media sequence) was the best of those tested in this study for shoot bud induction, though a minor improvement in shoot elongation and plant recovery was achieved by using the MS119 medium of Hyde and Phillips (1996) following the AZ medium. A combination of 250 mg l^{-1} ticarcillin and 250 mg l^{-1} claforan proved optimal for promoting tomato regeneration while suppressing *Agrobacterium* overgrowth, resulting in a significant improvement in transformation efficiency. Regeneration response was better when a lower kanamycin selection pressure (50 mg l^{-1}) was used during bud induction (AZ medium), while transformation efficiency was better when a higher level of kanamycin (150 mg l^{-1}) was used during shoot elongation (MS119 medium). Transgene inheritance was demonstrated in the T_1 generation through phenotype inheritance (GUS expression), and confirmed by PCR analysis. About three-fourths of the independent transformant lines tested showed Mendelian inheritance ratios, primarily as single-copy events but also including multiple-copy events; a majority of the remainder appeared to represent chimeric events.

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