Chapter 28

Tomato (*Solanum lycopersicum*)

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Abstract

Agrobacterium-mediated transformation is the most common method for the incorporation of foreign genes into the genome of tomato as well as many other species in the *Solanaceae* family. This chapter describes a protocol for the genetic transformation of tomato cultivar Micro-Tom using cotyledons as explants. Detailed procedures are also included for determining gene-copy number using a duplex qPCR TaqMan assay, and the histochemical analysis of GUS expression.

Key words *Agrobacterium tumefaciens*, β-Glucuronidase (*uidA*), Neomycin phosphotransferase II (*npt* II), Plant transformation, *Solanum lycopersicum*, Tomato Micro-Tom

1 Introduction

Agrobacterium-mediated transformation is a technology that has been used as an alternative to introduce genes that control different agronomical traits for crop improvement [1]. Tomato is the second most important vegetable crop throughout the world, with an annual production of 153 million metric tons in 2009 [2]. Since the first report on the genetic transformation of tomato [3], many studies have contributed to the advancement and improvement of tomato transformation methodology using *Agrobacterium* [4–9]. However, there are still limitations in the efficient genetic transformation of different tomato varieties [3]. With the completion of the full genome sequence of tomato [10], the development of highly efficient transformation methods is necessary to help understand gene function and regulation, and the development of new biotechnological products. Micro-Tom is being used as a model cultivar for functional genomics studies in tomato, because its small size and short life cycle allow it to be grown in small areas and the mature fruit can be easily harvested within 70–90 days after sowing [4, 8, 11–13]. This chapter describes an *Agrobacterium* transformation protocol of tomato cultivar Micro-Tom using cotyledons as explants, including *Agrobacterium* infection, calli

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induction, and shoot regeneration under kanamycin selection conditions. Protocols are also included for the growth of the primary transformants (T_0 plants) in the greenhouse for the production of T_1 seed, foreign gene copy number determination using a duplex qPCR TaqMan assay, and the histochemical analysis of the ß-glucuronidase (GUS) reporter-gene expression in transgenic plants. With the protocol presented here, a transformation efficiency of up to 65 % of treated cotyledon explants has been obtained using the *Agrobacterium* strain GV3101 [4]. An optimized tissue culture/shoot regeneration protocol is required to use this transformation protocol with other tomato cultivars. Following this transformation protocol, and the *Agrobacterium* strain EHA105, transformation efficiencies between 9 and 12 % have been previously obtained with the tomato varieties Moneymaker, UC-Davis 82, and Motelle (unpublished data, Plant Transformation Research Center, University of California, Riverside, CA—www.ptrc.ucr.edu).

2 Materials

2.1 Agrobacterium tumefaciens *Strains and the Binary Vector*

1. Recommended *A. tumefaciens* strains: GV3101 and EHA105. The gene of interest is cloned into a binary vector, and then transformed in *Agrobacterium* using electroporation.
2. Binary vector: The binary vector pBI121 (Clontech, Palo Alto Ca, USA) contains β-glucuronidase (*uidA*) and neomycin phosphotransferase II (*npt* II) genes under the regulation of the *CaMV* 35S promoter [14].

2.2 Plant Material

Tomato Micro-Tom seeds (Ball™ Seeds Co., Chicago, IL, USA).

2.3 Stock Solutions

All chemical ingredients for stock solutions and culture media can be obtained from different vendors including Sigma-Aldrich (www.sigmaaldrich.com), Plantmedia (www.plantmedia.com), Phytotechnology labs (www.phytotechlab.com), and Caisson laboratories (www.caissonlabs.com). Unless otherwise specified, all stock solutions are filter sterilized, and stored at −20 °C.

1. Nitsch vitamin solution (1,000×): To 80 mL of deionized water (dH_2O), add 200 mg glycine, 500 mg nicotinic acid, 50 mg thiamine–HCl, 50 mg pyridoxine–HCl, and 5 mg biotin. After all components are dissolved at room temperature (RT), adjust the volume to 100 mL. Nitsch vitamins include folic acid [15]. A folic acid 1,000× stock is prepared separately to avoid precipitates.
2. Folic acid solution (1,000×): Dissolve 50 mg of folic acid in 1 mL of 0.2 N NaOH. Bring up to 100 mL with dH_2O, and mix well. Because folic acid is light sensitive, it should be stored in a dark bottle at 4 °C.

3. Gamborg's B5 vitamins solution (1,000×): To 180 mL of dH_2O, add 200 mg myoinositol, 200 mg nicotinic acid, 200 mg pyridoxine hydrochloride, and 200 mg of thiamine hydrochloride. Bring up to 200 mL with dH_2O [16].
4. MS vitamin solution (1,000×): Dissolve 100 mg thiamine HCl, 50 mg pyridoxine HCl, 50 mg nicotinic acid, and 200 mg glycine in 100 mL of dH_2O [17].
5. Indole-3 butyric acid (IBA) solution (1 mg/mL): The IBA solution is prepared by dissolving 20 mg of IBA in 2 mL of 95 % ethanol. Bring up to 20 mL with dH_2O.
6. Zeatin solution (1 mg/mL): Dissolve 50 mg zeatin in 1 mL of 1 M NaOH. Bring up to 50 mL with dH_2O. Prepare 1 mL aliquots, filter sterilize, and store at −20 °C.
7. Acetosyringone solution (74 mM): Dissolve 145 mg acetosyringone (3',5'-dimethoxy-4′-hydroxyacetophenone) in 10 mL of 95 % ethanol.
8. Kanamycin sulfate solution (100 mg/mL): Dissolve 1 g of kanamycin sulfate in 5 mL dH_2O, vortex, and bring up to 10 mL with dH_2O.
9. Cefotaxime sodium salt solution (250 mg/mL): Dissolve 2.5 g of cefotaxime in 5 mL ddH_2O, vortex, and bring up to 10 mL with dH_2O.
10. Carbenicillin disodium salt solution (500 mg/mL): Dissolve 5 g of carbenicillin in 5 mL dH_2O, vortex, and bring up to 10 mL with dH_2O.
11. Rifampicin solution (25 mg/mL): Dissolve 250 mg of rifampicin in 5 mL dH_2O, vortex, and bring up to 10 mL with dH_2O.

2.4 Culture Media

The pH of all plant culture media is adjusted to pH 5.8 with 1 M KOH, and sterilized by autoclaving. The hormones are added prior to autoclaving with the exception of zeatin, which, as well as the antibiotics, is added after autoclaving, when the temperature of the medium has dropped to 55 °C. Sterile medium is poured into 100 × 15 mm petri dishes or magenta vessels in a laminar flow hood. All media (liquid or solid) can be stored for several weeks at 4 °C, but media with antibiotics is better to use it fresh.

1. YEP-*Agrobacterium* medium: 10 g/L yeast extract, 10 g/L peptone, 5 g/L NaCl, adjust pH to 7.2 with 1 M NaOH. For solid medium, add bacto agar (15 g/L) before autoclaving [18].
2. A*grobacterium* infection medium (AIM): 4.3 g/L MS salts, 30 g/L sucrose, 1 mL MS vitamin stock solution (1,000×) [17].
3. Seed germination medium (SGM): 2.3 g/L MS salts, 20 g/L sucrose, 1 mL Gamborg's B5 vitamins, pH 5.8, 0.8 % agar (tissue culture grade). Pour sterile media in magenta boxes (~100 mL/box) to germinate seeds.

4. Co-culture medium (CCM): 4.3 g/L MS salts, 30 g/L glucose, 1 mL Gamborg's B5 vitamins (1,000×), 1 mg/L zeatin, pH 5.8, 0.8 % agar. Pour sterile media in 100 × 15 mm petri dishes.
5. Tomato shoot induction medium (TSIM): 4.3 g/L MS salts, 30 g/L sucrose, 2 mg/L zeatin, 100 mg/L *myo*-inositol, 1 mL Nitsch vitamins (1,000×), 1 mL folic acid (1,000×), pH 5.8, 0.8 % agar. Pour sterile media in 100 × 15 mm petri dishes.
6. Tomato shoot elongation medium (TSEM): 4.3 g/L MS salts, 30 g/L sucrose, 100 mg/L *myo*-inositol, 1 mL Nitsch vitamins (1,000×), 1 mL folic acid (1,000×), pH 5.8, 0.8 % agar. Pour sterile media in magenta vessels.
7. Tomato rooting medium (TRM): 4.3 g/L MS salts, 30 g/L sucrose, 100 mg/L *myo*-inositol, 1 mL Nitsch vitamins (1,000×), 1 mL folic acid (1,000×), 2 mg/L IBA, pH 5.8, 0.8 % agar. Pour sterile media in magenta vessels.

2.5 DNA Isolation, PCR Reaction, and Electrophoresis

1. 1 M Tris–HCl pH 7.5: Dissolve 121.1 g Tris base in 800 mL of ddH_2O. Adjust the pH to 7.5 by adding approximately 70 mL of concentrated HCl. Adjust the volume of the solution to 1 L with ddH_2O. Store into aliquots and sterilize by autoclaving.
2. 5 M NaCl: Dissolve 146.1 g NaCl in 350 mL ddH_2O. Bring up to 500 mL with ddH_2O.
3. 0.5 M EDTA pH 8.0: Add 186.1 g of disodium EDTA to 800 mL of ddH_2O. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (~20 g of NaOH pellets). Bring up to 1 L with ddH_2O. Dispense into aliquots and sterilize by autoclaving (*see* **Note 1**).
4. 10 % Sodium dodecyl sulfate (SDS): Dissolve 100 g SDS in 800 mL H_2O. Place in 1 L bottle on a shaker until dissolved. Adjust pH to 7.2 with 0.5 N HCl. Bring up to 1 L. Sterilize by autoclaving.
5. Extraction buffer: Mix 10 mL 1 M Tris pH 7.5, 2.5 mL 5 M NaCl, 2.5 mL 0.5 M EDTA, and 2.5 mL 10 % SDS in a 50 mL Falcon tube. Bring up to 50 mL with ddH_2O. This buffer is stable at RT.
6. TAE buffer (50×): Dissolve 242 g of Tris base in 700 mL ddH_2O. Add 57 mL of glacial acetic acid and 100 mL 0.5 M EDTA (pH 8.0). Pour mixture into 1 L graduated cylinder and add ddH_2O to a final volume of 1 L. Dispense into a glass bottle and store at RT.
7. TE buffer: Mix 5 mL 1 M Tris–HCl pH 7.5 and 1 mL 0.5 M EDTA pH 8.0 in about 100 mL ddH_2O, and bring up to

500 mL with ddH_2O. Sterilize by autoclaving. Make 25 mL aliquots and store at 4 °C.

8. Loading buffer (6×): Dissolve 25 mg of bromophenol blue in 3 mL sterile ddH_2O, and 3 mL of glycerol, and bring up to 10 mL with ddH_2O. Store at 4 °C.
9. Ethidium bromide: Dissolve 10 mg in 1 mL of ddH_2O. Bring up to 10 mL. Dispense into a dark glass bottle and keep at RT (*see* **Note 2**).
10. 1 Kb plus DNA ladder (Invitrogen): Mix 67 µl of 6× loading buffer, 100 µl of DNA ladder, and 733 µl of TE. Store at −20 °C.

2.6 Histochemical Analysis

Gus staining is a convenient way to analyze the expression of *uidA* gene, which encodes the ß-glucuronidase (GUS). After staining for GUS expression, the tissue can be either examined as whole amount preparation or processed further to observe activity patterns in tissue sections using a microscope [19].

1. Chloramphenicol (25 mg/mL): Dissolve 25 mg of chloramphenicol in 1 mL 100 % ethanol. Store at −20 °C.
2. Phosphate buffer pH 7.0: Dissolve 2.76 g $NaH_2PO_4{\cdot}H_2O$ in 20 mL of ddH_2O. In a different beaker dissolve 9.38 g $Na_2HPO_4 \cdot 2H_2O$ dibasic in 35 mL ddH_2O (dissolves at 37 °C). Combine 20 mL monobasic solution with 15 mL dibasic solution. Adjust the pH to 7 with dibasic solution.
3. $K_3(FeCN_6)$ (0.5 M): Dissolve 82.5 mg in 500 µl ddH_2O.
4. $K_4(FeCN_6)$ (0.5 M): Dissolve 105.5 mg in 500 µl ddH_2O.
5. Triton X-100 (10 %): Dissolve 10 g of Triton X-100 in 80 mL of ddH_2O. Bring up to 100 mL and stir until well mixed. Sterilize by autoclaving and store at 4 °C.
6. X-gluc staining solution: Dissolve 100 mg of X-Gluc in 200 µl N N-dimethylformamide (DMF). Add more DMF until the solution is transparent.
7. GUS solution: Combine all the following ingredients: 800 µl Chloramphenicol (25 mg/mL), 200 µl 0.5 M $K_3(FeCN_6)$, 200 µl 0.5 M $K_4(FeCN_6)$, 4 mL 0.5 M EDTA pH 8.0, 200 µl 100 % Triton X-100, X-gluc solution. Bring up to 200 mL with ddH_2O. Filter solution with a 0.2 µm Millipore filter. Aliquot in 50 mL Falcon tubes (covered in foil), and store at −20 °C.

2.7 Other Solutions and Supplies

1. Seed sterilization solution: 10 % commercial bleach (5.25 % sodium hypochlorite) plus one drop of Tween-20 per 50 mL.
2. Sunshine Universal Mix soil: Fosters (Waterloo, IA).
3. Sterile paper plates and towels.

4. Greenhouse standard open flat with drainage hole: McConkey, Cat # EJPFONH. Clear Humi-dome 7″ (plastic, transparent): McConkey, Cat # HYFCKDOME-50.
5. Jiffy Peat Pellets 42 mm: McConkey, Cat # JPA703.
6. Round Euro Pot 16 cm (diameter 6.25", height 6.75", volume 2.5 qt/2.37 L), McConkey, Cat # JMCATRI100B.

3 Methods

3.1 Seed Preparation

1. Place the tomato seeds in a 50 mL sterile Falcon tube.
2. Add 40 mL of seed sterilization solution, and shake the tube for 20 min in a shaker at 50 rpm.
3. Remove the sterilization solution and wash the seeds five times with sterile ddH_2O.
4. In a laminar flow hood, plant the sterilized seeds on seed germination medium (SGM).
5. Incubate seeds for 7 days at 24 °C under fluorescent light at 60 μEM/m^2/s with a photoperiod of 16/8-h light/dark.

3.2 Preparation of Agrobacterium Culture

1. Follow standard techniques to transform *Agrobacterium* strains with a binary vector that carries the gene or genes of interest. The *Agrobacterium* strains GV3101 and EHA 105 are kept for a long term in glycerol stocks at −80 °C.
2. To initiate the transformation take a loopful of culture from a glycerol stock of *Agrobacterium* and streak it on YEP solid medium containing the appropriate antibiotics (*see* **Note 3**). Incubate the plates at 28 °C for 2 days.
3. Inoculate a freshly grown single colony of *Agrobacterium* in 2 mL YEP with antibiotics. Incubate the culture in a shaker (250 rpm) for 2 days at 28 °C.
4. Take 100 μL of liquid *Agrobacterium* culture to inoculate 50 mL YEP, and incubate for another 2 days at 28 °C with shaking.
5. Spin down the culture at 5,000 rpm (2,152 × *g*) for 10 min and resuspend the pellet in 10 mL of AIM.
6. Determine the OD_{600} nm of the *Agrobacterium* culture, and adjust the OD to 0.6 with AIM (*see* **Note 4**).
7. Add 20 μL of acetosyringone stock (74 mM) to 40 mL AIM-diluted *Agrobacterium* culture to be used for transformation.

3.3 Preparation of Explants for Agrobacterium Inoculation

1. Cotyledons are ready for transformation when they just emerge from the seed coat. The cotyledons should be dark green and the seedlings compact, not pale green and spindly (*see* Fig. 1a, b).

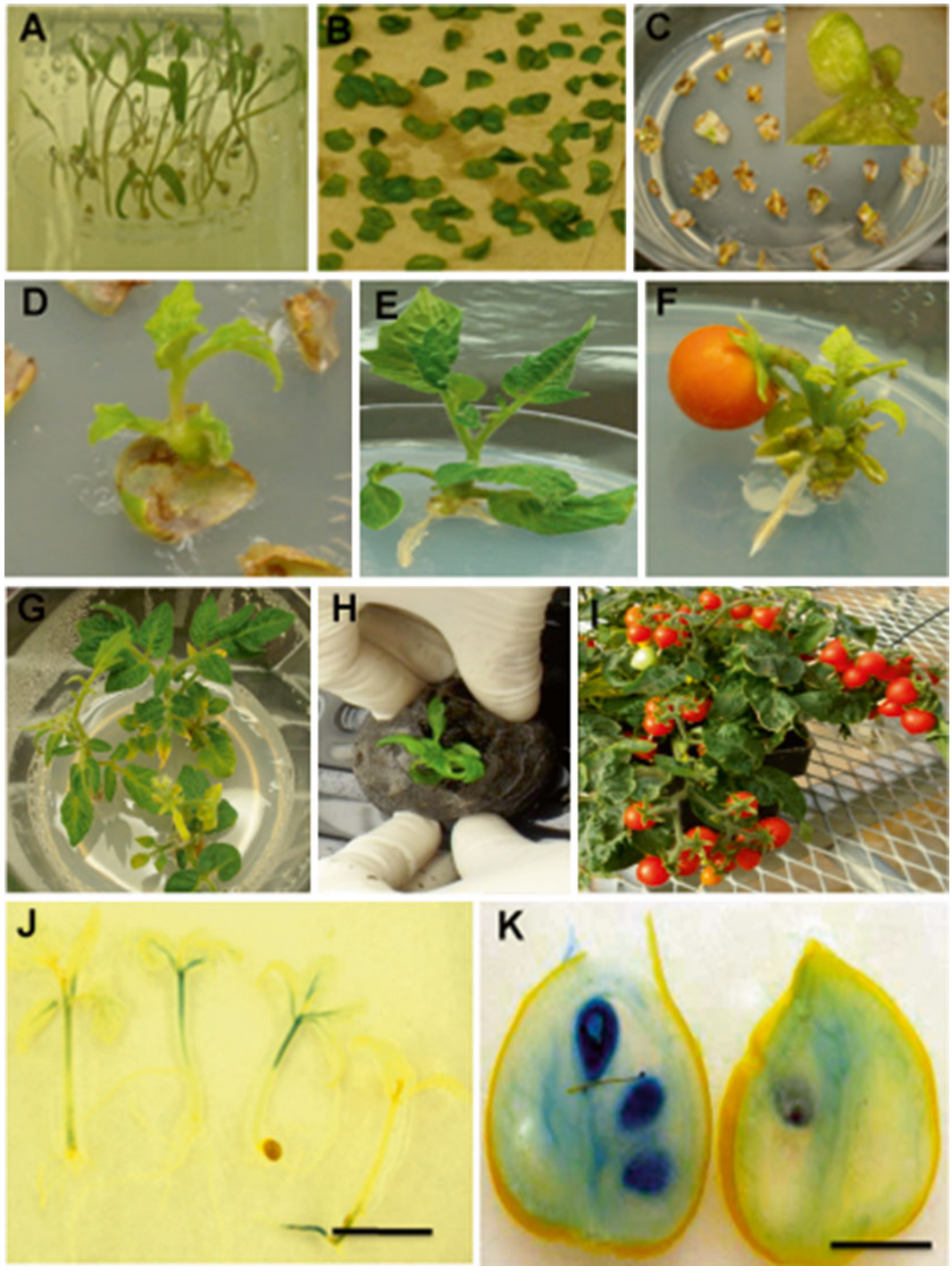

Fig. 1 Steps in the transformation of tomato cv. Micro-Tom with *Agrobacterium*. (**a**) Seed germination (1 week). (**b**) Blot drying of dissected cotyledon explants on sterile paper towels. (**c**) Shoot induction after 4 weeks on TSIM selection medium. (**d**) Shoot elongation in STEM for 2–4 weeks. (**e**, **f**) Rooting and plant development in TRM medium (6 weeks). (**g**, **h**) Transfer and acclimation of rooted shoots to soil (jiffy pots). (**i**) Fruit development for collection of transgenic T_1 seed in the greenhouse (3 months). (**j**, **k**). Histochemical detection of GUS expression in transgenic T_1 seedlings and tomato Micro-Tom fruit transformed with plasmid pBI121. Scale bars = 1.0 (**j**) and 2.0 cm (**k**). Total time for production of transgenic T_0 plants and collection of T_1 seeds is 6–8 months

2. Dissect cotyledons with a sharp scalpel blade in a sterile paper plate with humid paper towels. The base (1–2 mm) and the tip (1–2 mm) of the cotyledons are cut off, and then cut in halves (*see* Fig. 1b).
3. Transfer cotyledon explants abaxial side up onto Whatman filter paper placed over CCM medium in 100×15 mm petri dishes (*see* **Note 5**).
4. Seal the plates using plastic wrap, and incubate them for 2 days in a growth room at 24 °C, under fluorescent light (60 μE/m^2/s) with a photoperiod of 16/8-h light/dark.

3.4 Agrobacterium Infection and Cocultivation

1. Transfer cut cotyledon explants into a Falcon tube with 40 mL of the *Agrobacterium* suspension in AIM previously prepared (*see* **step 7** of Subheading 3.2).
2. Incubate the cotyledons with *Agrobacterium* cells for 20 min. Shake the tubes gently during the incubation time in a rotatory or horizontal shaker at 50 rpm.
3. Remove the *Agrobacterium* suspension and transfer the cotyledons onto sterile paper towels. Blot dry the cotyledons between two sterile filter papers or sterile paper towels (*see* **Note 6**).
4. Transfer the cotyledon explants abaxial side up onto a new 100×20 mm petri dish containing a Whatman filter paper placed on CCM (*see* **Note 5**).
5. Seal the plates using plastic wrap, and incubate the explants for 2 days as before (*see* **step 4** of Subheading 3.3).

3.5 Selection of Transgenic Callus and Shoot Induction

1. After 2 days of cocultivation, collect the cotyledons in Falcon tubes, and rinse them for 5 min with 40 mL of 250 mg/mL of cefotaxime in water. Blot dry the tissue explants between sterile filter paper or sterile paper towels and transfer them to TSIM containing 500 mg/L carbenicillin, 250 mg/mL cefotaxime, and 100 mg/L kanamycin (without filter paper). Explants are placed abaxial side up over the surface of the medium without filter paper (15–20 cotyledons per plate).
2. Seal the plates with plastic wrap and incubate them as before (*see* **step 4** of Subheading 3.3).
3. Transfer the explants to fresh TSIM plates two times every 2 weeks. If *Agrobacterium* overgrowth is observed, the explants should be washed again 3× with 250 mg/L cefotaxime, and 500 mg/L carbenicillin, blot dry again, and continue culturing on TSIM. Shoot primordia start to appear after the first 2 weeks (*see* Fig. 1c).

3.6 Shoot Elongation and Root Induction

1. Transfer explants with shoot primordia to shoot elongation medium (TSEM) supplemented with the same antibiotics (*see*

step 1 of Subheading 3.5) in magenta boxes, and incubate as before (*see* **Note 7**).

2. After 4 weeks on TSEM, transfer elongated shoots that are at least 2 cm long to magenta boxes containing 50 mL of TRM supplemented with 250 mg/L carbenicillin, 125 mg/L cefotaxime, and 50 mg/L kanamycin, and incubate them as before (*see* **Note 7** and Fig. 1d–f).

3.7 Transplanting and Acclimation of Rooted Shoots

1. Gently remove the shoots with well-formed root systems from the magenta boxes. Wash off the agar medium from the roots using ddH_2O.
2. Transfer each in vitro plantlet to a Jiffy peat pellet and place them in a flat tray with water in the greenhouse (*see* Fig. 1g, h). Use a transparent dome to cover the flat to maintain a high relative humidity for acclimation during the first 3 days (*see* **Note 8**).

3.8 Greenhouse (GH) Care of Transgenic Plants

1. After 1 week of acclimation, when roots are coming out of the jiffy peat pellet transfer the established tomato plantlet to 2″ pots with Sunshine Universal soil mix L, wet with regular water, and then move them to the greenhouse. Ensure that plants are accurately labeled. The ideal GH environmental conditions for Micro-Tom are temperature 22 °C, relative humidity ~70 %, and natural light conditions.
2. After 3 weeks in small pots, transfer the tomato plants to 6.5" pots with Sunshine Universal soil mix, and daily water them with nutrient water (e.g., half-strength Hoagland's solution).
3. Keep plants growing in the GH for around 6 months to fruit seed production (*see* Fig. 1i).

3.9 Transgenic Seed Production and Harvest

1. Harvest the T_0 fruit. As the fruit is perishable, collect the seeds as soon as possible. However, if many tomatoes are harvested, store the fruits at 4 °C for up to 2 weeks.
2. Collect the T_1 seeds by cutting the fruit in half and scraping the seeds into a blender with ~500 mL of water.
3. Stir the seeds for a few seconds, stop the blender, and let the seed go to the bottom of the glass. Then, discard the supernatant and drain the seeds. Spread the seeds over clean cheesecloth and allow them to dry for at least a week at RT.
4. Remove the seeds from the cheesecloth, weigh, and transfer them to appropriately labeled coin envelope.
5. Store the envelopes containing the dry T_1 seeds at 4 °C.

3.10 DNA Extraction and PCR Analysis

1. Grind 100 mg of leaves from putative transgenic (i.e., kanamycin resistant) in an Eppendorf tube with 450 μL of extraction buffer (200 mM Tris–HCl (pH 7.5), 250 mM NaCl, and

Table 1
Description of primers and probes for end PCR and TaqMan qPCR [4]

Gene				
Primer/probe	**Sequence (5′-3′)**	**Length (bp)**	**Annealing (°C)**	**Product size (bp)**
End-PCR				
*npt*II F1	GGATTGCACGCAGGTTCTCC	20	55	532
*npt*II R1	AACTCGTCAAGAAGGCGATA	20		
TaqMan qPCR				
*npt*II F2	ATCCATCATGGCTGATGCAATGCG	24	60	81
*npt*II R2	CGATGTTTCGCTTGGTGGTCGAAT	24		
*npt*II probe —HEX	TGCATACGCTTGATCCGGCTACCT	24		
*uid*A F2	TCCATCGCAGCGTAATGCTCTACA	24	60	100
*uid*A R2	TCAACAGACGCGTGGTTACAGTCT	24		
*uid*A probe —HEX	ACGATATCACCGTGGTGACGCATGT	25		
Prosys F1	GTGACGTGAAAGCAATATCAAGAGCCC	27	60	117
Prosys R1	CGCGCATTATGTTGAGATGTGTGC	24		
Prosys probe —FAM	TCTTTCTTCTCGTGAAGTATAGG AGCGCT	29		

25 mM EDTA (pH 8.0), 0.5 % SDS), followed by chloroform extraction, and isopropanol precipitation of nucleic acids.

2. Wash DNA pellet with 70 % ethanol two times, and resuspend in 50 µL of TE buffer.
3. Resuspend DNA at a final concentration of 20 ng/µL using nuclease-free water.
4. For regular end-PCR analysis, 100 ng of DNA is added to a 20 µl PCR reaction mix containing 0.25 mM dNTPs, 2 mM $MgCl_2$, 1 U ExTaq DNA polymerase (Life Technologies®, NY, USA), and 0.5 µM of each primer pair for the amplification of the *nptII* gene (*see* Table 1).
5. Reaction conditions for end PCR are 94 °C for 5 min, followed by 29 cycles of 94 °C for 30s, 60 °C for 30s, 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR products are visualized after electrophoresis on 0.8 % agarose gels. The gel is scored for the presence or absence of the *npt*II product (532 bp).

3.11 qPCR Assay to Estimate Copy Number

1. The comparative qPCR (ΔΔCt) technique for duplex TaqMan reactions can be used to determine foreign gene copy number in transgenic plants, using the tomato prosystemin gene [20] as an endogenous, single-copy reference gene [4].
2. Optimal TaqMan assay efficiencies for the gene target (e.g., nptII or uidA), and the reference prosystemin gene primers and probe sets are determined from serial dilutions of template DNA (*see* Table 1; [4, 21].
3. Duplex qPCR reactions are performed in triplicate 25 μl volumes using iQ SensiMix IITM Probe Master mix (Bio-Rad Laboratories, Hercules, CA, USA), 400 nM of each gene-specific primer, 200 nM of specific TaqMan probe, and 25 ng of sample DNA. The qPCR protocol includes 10-min activation step at 95 °C, followed by 40 cycles featuring a 10-s denaturation step at 95 °C, and a 1-min annealing and extension step at 60 °C. Data acquisitions are done at 72 °C as the machine ramp from annealing/extension to denaturation.
4. Sample Ct values are determined and target/reference ratios are normalized against a known positive control containing one copy of the transgene (e.g., nptII or uidA), determined by Southern blot analysis [21]. Normalized copy number clusters around values of 1, 2, 3, and more copies.

3.12 Southern Blot Analysis

Genomic DNA from selected PCR-positive plants transformed with each *Agrobacterium* strain is used for Southern blot analysis. DNA from wild-type (non-transformed) Micro-Tom plants is used as a negative control. 5 μg of DNA from each plant is digested with restriction endonucleases (New England Biolabs®Inc, Ipswich, MA, USA), fractionated on 0.8 % agarose gels, transferred to nylon membranes, and hybridized with P^{32}-radioactive probes to detect either the *nptII* or the *uidA* genes by autoradiography [22].

3.13 Histochemical Analyses for GUS Assay

1. Submerge tissue samples in GUS solution. Keep samples on ice.
2. Place the tissue under vacuum for 10 min. Close the valve and let it stay for another 20 min in the dark.
3. Slowly open the valve and release the vacuum. Cover the container with parafilm, then wrap it in aluminum foil, and incubate at 37 °C for 8–16 h. Incubation time depends on the tissue and the promoter fusion being used.
4. Remove the container from the incubator, replace the GUS solution with 50 % ethanol, and incubate at 37 °C for 1 h. Repeat with 70 and 100 % ethanol until the tissue is cleared. Tissue can remain in 70–100 % ethanol indefinitely at 4 °C.
5. Look at the tissue samples under a microscope and take pictures (*see* Fig. 1j, k).

4 Notes

1. The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to ~8.0 by the addition of NaOH.
2. Ethidium bromide is a strong mutagen and a possible carcinogen or teratogen. Its hazardous properties require to use gloves for handling and safety disposal.
3. Rifampicin (25 mg/L) and kanamycin (50 mg/L) are added to grow *Agrobacterium* strains GV3101 and EHA105 transformed with pBI121.
4. To calculate the final dilution volume of *Agrobacterium* (V_1) use the equation $C_1V_1 = C_2V_2$, where C_1 and C_2 are, respectively, the initial and final OD concentration, and V_2 the final volume. Use AIM to dilute the *Agrobacterium* cells. Diluted *Agrobacterium* culture has to be used immediately to avoid overgrowth and aggregation.
5. Since the cotyledons are very fragile, handle them carefully but quickly to avoid dehydration. A sterile Whatman filter paper placed over the medium is used to avoid overgrowth of *Agrobacterium* during the cocultivation time. Transfer 20–30 cotyledons per plate. This step is very critical to avoid overgrowth of *Agrobacterium* and obtain good transformation efficiencies. Be careful with the handling of the tissue because the explants are very fragile. There is a high correlation between the transformation efficiency and this particular feature.
6. Shoots regenerated from different sectors of the explant can be considered independent events.
7. Cut off the calli at the base from the shoot. Transgenic shoots start rooting after 10–15 days (*see* Fig. 1e).
8. Cover each plant immediately after transfer to soil to prevent wilting.

References

1. James C (2011) Global status of commercialized Biotech/GM crops: ISAAA Brief No. 43. ISAAA, Ithaca, NY
2. Krylod A (2012) UNCTAD http://www.unctad.info/en/Infocomm/AACPproducts/COMMODITY-PROFILE—Tomato/. Accessed on 19 April 2013
3. McCormick S, Niedermeyer J, Fry J, Barnason A, Horsh R, Fraley R (1986) Leaf disc transformation of cultivated tomato (*L. esculentum*) using *Agrobacterium tumefaciens*. Plant Cell Rep 5:81–84
4. Chetty VJ, Ceballos N, García D, Narváez-Vásquez J, López W, Orozco-Cárdenas ML (2013) Evaluation of four *Agrobacterium tumefaciens* strains for the genetic transformation of tomato (*Solanum lycopersicum L.*) cultivar Micro-Tom. Plant Cell Rep 32:239–247
5. Guo M, Zhang YL, Meng ZJ, Jiang J (2012) Optimization of factors affecting *Agrobacterium*-mediated transformation of Micro-Tom tomatoes. Genet Mol Res 11:661–71
6. Hamza S, Chupeau Y (1993) Re-evaluation of conditions for plant regeneration and

Agrobacterium-mediated transformation from tomato (*Lycopersicon esculentum*). J Exp Bot 44:1837–1845

7. Park SH, Morris JL, Park JE, Hirschi KD, Smith RH (2003) Efficient and genotype-independent *Agrobacterium* – mediated tomato transformation. J Plant Physiol 160: 1253–1257
8. Sun HJ, Uchii S, Watanabe S, Ezura A (2006) A highly efficient transformation protocol for Micro-Tom, a model cultivar for tomato functional genomics. Plant Cell Physiol 47:426–431
9. Van E, Kirk DD, Walmsley A (2006) Tomato (*Lycopersicon esculentum*) (Chapter 39). In: *Agrobacterium* protocols (3rd ed). Humana, Totowa, NJ, pp 459–473
10. Sato S, Tabata S, Hirakawa H, Asamizu E, Shirasawa K et al (2012) The tomato genome sequence provides insight into fleshy fruit evolution. Nature 485:635–641
11. Dan Y, Yan H, Munyikwa T, Dong J, Zhang J, Armstrong CL (2006) Micro-Tom a high-throughput model transformation system for functional genomics. Plant Cell Rep 25: 432–441
12. Mathews H, Clendennen KS, Caldwell GC, Liu XL, Connors K, Matheis N, Schuster KD, Menasco DJ, Wagoner W, Lightner J, Wagner DR (2003) Activation tagging in tomato identifies a transcriptional regulator of anthocyanin biosynthesis, modification, and transport. Plant Cell 15:1689–1703
13. Meissner R, Jacobson Y, Melamed S, Levyatuv S, Shalev G, Ashir A, Elkind Y, Levy A (1997) A new model system for tomato genetics. Plant J 12:1465–1472
14. Po-Yen C, Chen-Kuen W, Shaw-Ching S, Kin-Ying T (2003) Complete sequence of the binary vector pBI121 and its application in cloning T-DNA insertion from transgenic plants. Mol Breed 11:287–293
15. Nitsch JP, Nitsch C (1969) Haploid plants from pollen grains. Science 163:85–87
16. Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158
17. Murashige T, Skoog F (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol Plant 15:473–497
18. Hellen R, Mullineaux P (2000) A guide to *Agrobacterium* binary Ti vectors an update. Trends Plant Sci 5:446–451
19. Jefferson RA, Kavanagh TA, Bevan MW (1987) *GUS* fusion: *glucuronidase* as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907
20. McGurl B, Pearce G, Orozco-Cárdenas ML, Ryan CA (1992) Structure, expression and antisense inhibition of the system in precursor gene. Science 225:1570–1573
21. Ingham DJ, Beer S, Money S, Hansen G (2001) Quantitative real time PCR assay for determining transgene copy number in transformed cells. Biotechniques 31:132–140
22. Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, vol 3 (3rd ed), CSHL Press, New York, NY, pp 1–1453