Chapter 16

Agrobacterium tumefaciens-Mediated Transformation of Tomato

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Abstract

Tomato is both an important food crop and serves as a model plant species that is used for various research investigations including understanding gene function. Transformation is commonly utilized to facilitate these investigations in combination with all the extensive genetic and genomic resources available for tomato. The transformation protocol routinely used in our laboratory has been applied to many different tomato genotypes and relies on *Agrobacterium tumefaciens* infection of young cotyledon sections. We have used vector systems for overexpression, RNA interference for gene silencing, and CRISPR/Cas9 for genome editing. Vectors used to design gene constructs contained selectable marker genes that conferred resistance to kanamycin, hygromycin, and the herbicide component, bialaphos. The protocol we follow for *Agrobacterium*-mediated transformation of both cultivated and wild species of tomato is detailed in this chapter.

Key words AGL1, *Agrobacterium tumefaciens*, LBA4404, *Solanaceae*, *Solanum lycopersicum*, *Solanum pimpinellifolium*

1 Introduction

Tomato, a member of the *Solanaceae* family, is native to South America and was brought to Europe in the 1500s and then to North America in the 1800s [1]. It is a perennial plant that has determinate and indeterminate plant architecture where determinate is a compact growth habit, and indeterminate is a sprawling type of habit. There are two different market types of tomatoes, fresh market and processing. According to the Agricultural Marketing Resource Center, in 2014 the US dollar value for fresh market tomatoes was 1.14 billion and 1.325 billion for processing types, which are used to make products such as juice, sauces, and ketchup [2].

In addition to being an important food crop, tomato has also been exploited as a model species for various studies. Over the years, utilization of tomato as a model plant species has increased

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because of readily available resources such as mutant populations, bioinformatics tools [3], a high-quality reference genome [4], and efforts to improve *Agrobacterium*-mediated transformation efficiency and recovery time of transgenic lines [5, 6]. The first report of *Agrobacterium tumefaciens*-mediated transformation of tomato (*Solanum lycopersicum*) was by McCormick et al. [7] more than 30 years ago. In the intervening years, there have been reports of transformation of different tomato genotypes and different explant types such as cotyledons, hypocotyls, and leaves [5, 8–13]. Availability of efficient transformation methods is especially critical with the rapid development of genome editing technologies, which will result in an increased demand for generation of transgenic lines for basic research studies that can lead to crop improvement.

The methodology reported in this chapter is based on a modified version reported by Fillatti et al. [9] for infection of tomato cotyledons with *Agrobacterium* (Fig. 1). Briefly, our method starts with disinfected seeds cultured on a medium for germination. Cotyledons collected before the first true leaves emerge are sectioned and infected with *Agrobacterium tumefaciens* followed by a cocultivation period of 2 days. The cotyledon sections are transferred to a selective regeneration medium that contains selection

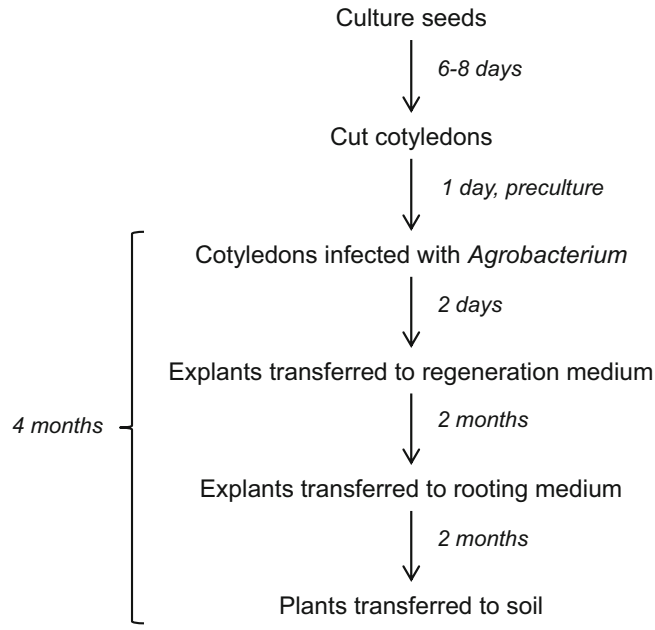

Fig. 1 Tomato transformation workflow. Estimated time is for best-case scenario to recover the first rooted plant. Duration and efficiency of transformation are dependent on the gene of interest and genotype. Procedure from time of infection to soil transfer can take up to 6 months to recover ten rooted plants as plants regenerate at different time points during the process of generating transgenic lines

agents specific for the selectable marker gene in the vector used to build a construct. We have continued to build and optimize our method since the first report in 2006 [13], including the removal of a *Nicotiana tabacum* cell suspension (NT1) feeder layer during cocultivation. In a recent study, we showed that the time for recovery of transgenic lines from the tomato cultivar M82 can be significantly decreased by the addition of indole-3-acetic acid (IAA) to the regeneration medium [5]. However, we have not systematically tested this method on other genotypes; therefore, our recommendation is that it be tested in comparison with medium not containing IAA before adopting this as a standard medium component. Revisiting protocols for generation of stable transgenic lines to determine if methods could be made more efficient or less genotype dependent should always be considered in order to alleviate bottlenecks that prevent progress for investigations that rely on transformation [14].

2 Materials

2.1 Plant Material, Agrobacterium tumefaciens, and Vectors

1. One gram of seed of tomato genotype of interest (*see* **Note 1**).
2. *Agrobacterium tumefaciens* strain AGL1 or LBA4404 (*see* **Note 2**).
3. Vectors commonly used for our work include but are not limited to p201N:Cas9 and pAGM4723 for CRISPR/Cas9-mediated gene editing, pHellsgate8 for RNAi-mediated silencing, and pBTEX for overexpression.

2.2 Media Components and Stock Solutions (See Note 3)

1. 1 mg/mL Thiamine HCl: Dissolve 50 mg of thiamine HCl in 50 mL deionized H_2O. Wrap in foil and store at 4 °C.
2. 0.5 mg/mL Pyridoxine HCl: Dissolve 25 mg of pyridoxine HCl in 50 mL deionized H_2O. Store in 1 mL aliquots at −20 °C.
3. 0.5 mg/mL Nicotinic acid: Dissolve 25 mg of nicotinic acid in 50 mL deionized H_2O. Store in 1 mL aliquots at −20 °C.
4. 1000× Modified Nitsch vitamins: Dissolve 0.1 g of glycine, 0.5 g of nicotinic acid, 0.025 g of pyridoxine HCl, 0.025 g of thiamine HCl, 0.025 g folic acid, and 0.002 g of d-biotin in 50 mL deionized H_2O. Adjust the pH to 7.00. Store in 1 mL aliquots at −20 °C.
5. 1 mg/mL trans-Zeatin: Dissolve 50 mg of trans-Zeatin in a few drops of 0.5 M HCl. Add deionized H_2O to a total volume of 50 mL. Filter sterilize and store in 1 mL aliquots at −20 °C. Add after autoclaving.
6. 100 mg/mL Timentin: Dissolve 5.17 g of timentin in 50 mL of deionized H_2O (*see* **Note 4**). Filter sterilize and store in aliquots at −20 °C. Add after autoclaving.

7. 100 mg/mL Kanamycin: Dissolve 5 g of kanamycin (Caisson Labs, Smithfield, UT, USA, product #K003) in 50 mL of deionized H2O. Filter sterilize and store in 1 mL aliquots at −20 °C. Add after autoclaving.
8. 50 mg/mL Spectinomycin: Dissolve 2.5 g of spectinomycin in 50 mL of deionized H_2O. Filter sterilize and store in 1 mL aliquots at −20 °C. Add after autoclaving.
9. 100 mg/mL Carbenicillin: Dissolve 5 g of carbenicillin in 50 mL of deionized H_2O. Filter sterilize and store in 1 mL aliquots at −20 °C. Add after autoclaving.
10. 12 mg/mL Rifampicin: Dissolve 120 mg of rifampicin in 10 mL of methanol. Wrap in foil and store at −20 °C.

2.3 Culture Medium

The media components listed are on a per-liter basis. Cool the medium in a water bath to 55–60 °C. This preserves the integrity of the hormones and antibiotics added after autoclaving while keeping the media from solidifying before poured. Dispense 24.5 mL of medium per Petri plate. For Magenta™ GA-7 boxes (referred to as Magenta boxes throughout the remainder of the chapter), we dispense 62.5 mL per Magenta box.

2.3.1 For Agrobacterium AGL1

1. MG/L bacterial medium: 5 g of tryptone, 2.5 g of yeast extract, 5 g NaCl, 5 g of mannitol, 0.1 g of $MgSO_4$, 0.25 g of K_2HPO_4, 1.2 g of glutamic acid, 15 g of sucrose, pH to 7.2, 15 g Bacto™ Agar. After autoclaving, add 0.5 mL of carbenicillin stock and antibiotics appropriate for vector. Dispense into 100 × 15 mm Petri plates.
2. Luria Broth (LB): 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, and antibiotics appropriate for vector.

2.3.2 For Agrobacterium LBA4404

1. LB bacterial medium: 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, 15 g of Bacto™ Agar. After autoclaving, add 1.25 mL rifampicin stock and antibiotics appropriate for vector. Dispense into 100 × 15 mm Petri plates.
2. YEP liquid: 10 g of yeast extract, 10 g of peptone, 5 g of NaCl, and antibiotics appropriate for vector.

2.3.3 For Seed Germination and Transformation

1. Seed germination medium (½ MSO): 2.15 g of Murashige and Skoog (MS) salts, 100 mg of myoinositol, 2 mL of thiamine HCl stock, 1 mL of pyridoxine HCl stock, 1 mL of nicotinic acid stock, 10 g of sucrose, pH to 5.8, 8 g agar (Millipore Sigma, Burlington, MA, USA, product #A1296). Dispense 50 mL per Magenta box.
2. Liquid coculture medium (2% MSO): 4.3 g of MS salts, 100 mg of myoinositol, 0.4 mL of thiamine HCl stock, 20 g of sucrose, pH to 5.8.

3. Preculture/coculture medium (2Z preculture): 4.3 g of MS salts, 100 mg of myoinositol, 1 mL of modified Nitsch vitamin stock, 20 g of sucrose, pH to 6.0, 5.2 g of TC Gel (Caisson Labs, Smithfield, UT, USA, product #PTP02). After autoclaving, add 2 mL of trans-zeatin stock. Dispense into 100 × 20 mm Petri plates (*see* **Note 5**).

2.3.4 For Plant Regeneration and Rooting

1. Primary regeneration medium (2Z): 4.3 g of MS salts, 100 mg of myoinositol, 1 mL of modified Nitsch vitamin stock, 20 g of sucrose, pH to 6.0, 5.2 g of TC Gel. After autoclaving, add 2 mL of trans-zeatin stock, 3.5 mL of timentin stock, and antibiotics appropriate for vector. Dispense into 100 × 20 mm Petri plates (*see* **Note 5**).
2. Secondary regeneration medium (1Z): Same as 2Z but with only 1 mL of trans-zeatin stock. Dispense into 100 × 20 mm Petri plates (*see* **Note 5**) and Magenta boxes.
3. Rooting medium (RM): 4.3 g of MS salts, 1 mL of modified Nitsch vitamin stock, 30 g of sucrose, pH to 6.0, 8 g Bacto™ Agar. After autoclaving, add 3.5 mL of timentin stock and antibiotics appropriate for vector. Dispense into Magenta boxes.

2.4 Other Supplies and Reagents

1. Sterile deionized water.
2. Sterilization solution (20% bleach plus one drop Tween 20).
3. Sterile Whatman filter paper, 7 cm in diameter.
4. Pipetman and sterile tips.
5. Sterile paper towels.
6. 100 × 15 mm and 100 × 20 mm sterile Petri plates.
7. 0.5 in Micropore tape (Fisher Scientific, Birmingham, AL, USA, product #19-061655).
8. Parafilm.
9. 50 mL Falcon disposable centrifuge tube, or similar.
10. Magenta™ GA-7 boxes (referred to as Magenta boxes in this chapter).

3 Methods

3.1 Preparation of Plant Material

1. Sterilize seed by immersing 0.9–1.0 g seeds in 25 mL sterilization solution and agitate for 20 min at 250 rpm on an orbital shaker. Rinse three times with sterile deionized H_2O.
2. Place 25–30 seeds per Magenta box containing ½ MSO (Fig. 2a).

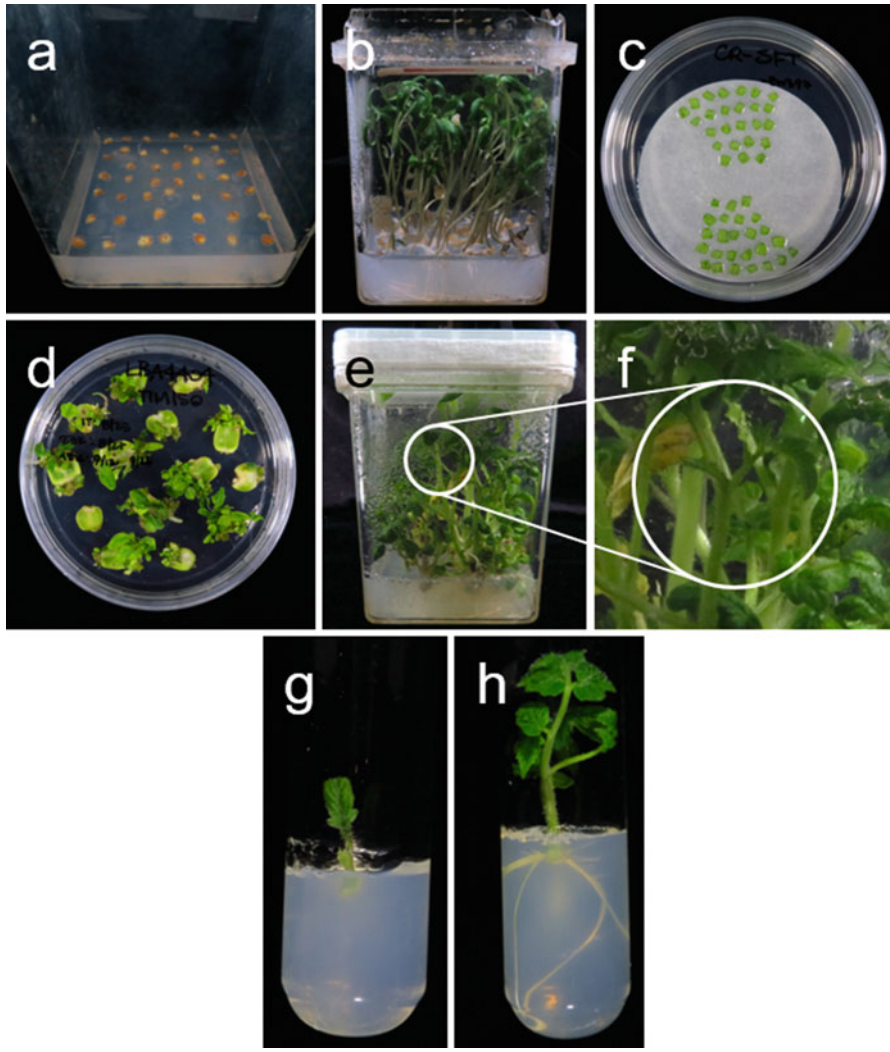

Fig. 2 Generation of transgenic tomato lines from cotyledon explants. Explants were infected with *Agrobacterium tumefaciens* that contained a plasmid harboring the selectable marker gene neomycin phosphotransferase II (*nptII*) gene which confers resistance to kanamycin. (**a**) Tomato seeds placed in Magenta boxes containing ½ MSO. (**b**) 7-Day-old seedlings grown on ½ MSO medium. (**c**) Cotyledons cut and placed adaxial (top) side down on sterile filter paper in Petri plates containing 2Z preculture medium. (**d**) Explants on 1Z selective medium containing kanamycin. (**e**) Putative transgenic lines on first phase of RM selective medium. (**f**) Close-up of nodal region ready for excision and transfer into test tube containing RM. (**g**) Excised shoot in test tube containing RM selective medium. (**h**) Rooted shoot, 2 weeks after transfer into tube containing RM selective medium

3. Cut cotyledons from 6- to 8-day-old seedlings (Fig. 2b) (*see* **Note 6**), before the first true leaves have appeared (*see* **Note 7**). Place seedling on a sterile paper towel moistened with sterile deionized H_2O. Excise cotyledons at petiole and cut off tips. Cut cotyledons into 1 cm sections.

4. Place cut cotyledons adaxial (top) side down on plates of 2Z preculture medium, 50 pieces per plate (Fig. 2c). A piece of sterile Whatman filter paper is laid on top of the preculture medium and moistened with sterile water. For controls, ten pieces are used (five for positive controls, five for negative controls).
5. Seal plates with Parafilm. Culture for 1 day at 24 °C ± 2 °C with a 16-h photoperiod.

3.2 Transformation and Selection of Transgenic Lines

1. Two to three days before transformation, streak *Agrobacterium* containing the construct of interest onto selective MG/L or LB bacterial medium. Incubate for 36–48 h at 28 °C until colonies have developed.
2. Select four single, well-formed colonies from the plate and transfer to 50 mL LB or YEP medium containing antibiotic selection appropriate for the vector. Culture in a shaking incubator at 250 rpm at 28 °C overnight.
3. Check the OD_{600}. Optimum OD_{600} is 0.6. If OD_{600} is more than 0.65, dilute the culture until the OD_{600} reading is below 0.5 and grow for another hour. Check the OD_{600} reading periodically.
4. Centrifuge at 8228 × *g* for 10 min at 20 °C.
5. Note and record the volume of the supernatant. Discard the supernatant. Add 10 mL 2% MSO.
6. Resuspend the pellet by vortexing three times for 3–4 s each time. Then bring up to the volume recorded in **step 5** with 2% MSO.
7. Pipette 25 mL *Agrobacterium* culture into a sterile Magenta box. Transfer explants from 2 plates into inoculum (100 explants per 25 mL culture). Incubate explants for 5 min with occasional shaking. For controls, 25 mL of 2% MSO is used.
8. Remove explants to a sterile paper towel. Return explants adaxial side down to plates containing 2Z preculture medium. Seal plates with Parafilm.
9. Maintain plates under dark conditions at 19 °C for 48 h.
10. Transfer 25 explants to each plate of 2Z selective medium, adaxial side up. For controls, transfer five explants to 2Z medium without a selective agent (positive controls), and the other five explants to 2Z medium with selective agent (negative controls). Seal plates with Micropore tape. Culture at 24 °C ± 2 °C, with a 16-h photoperiod for 2 weeks.

11. Transfer to fresh 1Z selective medium (15 explants per plate), and to freshly prepared medium every 2 weeks (Fig. 2d) (*see* **Note 8**). When shoots begin to appear and touch the lid of the plate, transfer explants to 1Z selective medium in Magenta boxes, five explants per Magenta box (*see* **Note 9**).

3.3 Regeneration and Rooting

1. Initial shoots should appear within 4–6 weeks (Fig. 2e).
2. Excise shoots from explants when shoots are at least 2 cm and include at least one node (Fig. 2f). Place in Magenta boxes containing RM selective medium, four shoot cuttings per Magenta box (*see* **Note 9**). Tubes containing RM selective medium can also be used, with one shoot cutting per tube (Fig. 2g) (*see* **Note 10**).
3. Roots should begin to appear in 5–9 days (Fig. 2h).

4 Notes

1. Various tomato genotypes have been transformed with this protocol including M82, Rio Grande, Castlemart, cherry tomatoes including Sweet100, and the wild species *S. pimpinellifolium*.
2. There are some differences between *Agrobacterium tumefaciens* strains LBA4404 and AGL1. Transgenics generated with either strain generally contain a low copy number of the introduced transgene as compared to other *Agrobacterium* strains. AGL1 is a hypervirulent strain, and we have observed faster growth compared to LBA4404 with both solidified and liquid media. There are also more incidences of *Agrobacterium* AGL1 overgrowth in tissue culture postinfection as compared to LBA4404. Therefore, we recommend using 350 mg/L timentin in all culture media for AGL1 while 300 mg/L timentin is sufficient when using LBA4404. Overall, we see higher transformation efficiency with AGL1 (unpublished data), possibly because of its hypervirulence. Also, being that AGL1 is *recA*$^{-}$, there is less chance of the occurrence of recombination of introduced plasmid DNA [15].
3. In general, we keep refrigerated stock solutions for up to 3 months and frozen stock solutions for up to 6 months; however, this can be dependent on the stability of individual compounds. For frozen stock solutions, do not exceed two freeze/thaw cycles, as this impacts efficacy of the solution.
4. We have used timentin from bioWORLD, and GoldBio with similar effectiveness. Bioworld gives slightly higher transformation efficiency but results are not significant (unpublished data).

5. We use 100 × 20 mm Petri plates because they provide more space compared to 100 × 15 mm plates, allowing more room for shoots to expand. This additional space of the deeper plates has the potential to lessen the negative effects of accumulated ethylene, which can inhibit growth.
6. Time of germination, synchrony, and percentage vary depending on the tomato genotype.
7. Using cotyledons from seedlings after the first true leaves have appeared dramatically decreases transformation efficiency.
8. Media in Petri plates and Magenta boxes kept at room temperature are good for 2 weeks. If stored at 4 °C, they can be used for up to a month. Decreased timentin efficacy in older media allows *Agrobacterium* overgrowth, leading to losses of plant material.
9. It is important to not exceed five shoots per Magenta box containing 1Z selective medium and only four shoots per Magenta box containing RM selective medium. We have observed significant negative effects on growth when these numbers are exceeded. Excised shoots can be rooted on medium in either Magenta boxes or test tubes. For shipping, we recommend test tubes because the medium is more secure and will not detach from the bottom as it does in Magenta boxes.
10. Excised shoots can be rooted in Magenta boxes or test tubes. For shipping, we recommend test tubes because the medium will not detach from the bottom as it does in Magenta boxes.

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