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Organogenesis From Transformed Tomato Explants

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Summary

Tomato was one of the first crops for which a genetic transformation system was reported involving regeneration by organogenesis from *Agrobacterium*-transformed explants. Since the initial reports, various factors have been studied that affect the efficiency of tomato transformation and the technique has been useful for the isolation and identification of many genes involved in plant disease resistance, morphology and development. In this method, cotyledon explants from in vitro-grown seedlings are precultured overnight on a tobacco suspension feeder layer. The explants are then inoculated with *Agrobacterium* and returned to the feeder layer for a 2-d period of cocultivation. After cocultivation, the explants are transferred to an MS-based selective regeneration medium containing zeatin. Regenerated shoots are then rooted on a separate selective medium. This protocol has been used with several tomato cultivars and routinely yields transformation efficiencies of 10–15%.

Key Words: *Agrobacterium tumefaciens*; biotechnology; gene transfer; genetic engineering; genetic transformation; *Lycopersicon esculentum*; morphogenesis; regeneration; transgenic plants.

1. Introduction

Organogenesis from transformed explants is a very common method for generating transgenic plants. This technique is often favored because, unlike embryogenesis, morphogenesis from organ explants has been developed for many different plant species. Thus, established organogenesis systems are often easily adapted into genetic transformation protocols. In addition, regeneration from pieces of nonmeristematic transformed tissue may present a lower risk of chimerism than regeneration from plant meristems. The technique was first popularized by Horsch et al. ***(1)***, who described a simplified method for *Agro-*

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bacterium-mediated transformation of plants. Since then, organogenesis has become the preferred method for the regeneration of transgenic individuals in many species including tobacco, petunia, tomato, potato, cauliflower, squash, cotton, chrysanthemum, sunflower, and apple. This chapter uses *Agrobacterium tumefaciens*-mediated transformation of tomato, *Lycopersicon esculentum*, to illustrate the methods for organogenesis from transformed explants.

The earliest reports of *Agrobacterium*-mediated transformation of tomato were by Horsch et al. ***(1)*** and McCormick et al. ***(2)*** nearly 20 yr ago. Other methods for introducing foreign DNA into the crop, including microinjection ***(3)***, particle bombardment ***(4,5)*** (*see* Chapter 4), electroporation, and polyethylene glycol (PEG)-mediated transformation of protoplasts ***(6,7)*** (*see* Chapter 5), have also been described. However, none of these techniques has proven to be as popular as the *Agrobacterium*-mediated method, which is favored for its practicality, effectiveness, and efficiency.

The ability to genetically engineer tomato has been of great value because of its agronomic and economic importance and its usefulness as a model system. Tomato was one of the first crop species with a molecular genetic map ***(8)*** and, more recently, is the subject of new areas of study including functional genomics, proteomics, and metabolomics. Genetic transformation of tomato played an integral role in the map-based cloning of the first disease resistance ***(9)*** and quantitative trait ***(10)*** loci isolated in plants and in the identification of genes for many other important agronomic, morphological, and developmental traits (e.g., *see* **refs. *11–13***). *Agrobacterium*-mediated transformation of tomato with an antisense polygalacturonase construct was also used to develop the first commercial transgenic plant product, the Flavr Savr tomato ***(14)***. In addition, the technique has been used to introduce very large fragments of DNA (up to 150 kb) into the tomato genome ***(15)***.

Since the early reports of transformation, several groups have described various factors that affect the efficiency of tomato transformation. Conditions that influence tomato transformation include the choice and age of explants ***(16–18)***, the length of preculture ***(18,19)***, the strain and concentration of the *Agrobacterium tumefaciens* culture used for cocultivation ***(17,19)***, the length of cocultivation and medium used ***(17)***, the use of a petunia or tobacco suspension culture feeder layer ***(17–20)***, the orientation (adaxial side up vs abaxial side up) of cotyledon explants ***(21)***, the gelling agent ***(21)***, the plate sealant ***(21)***, and the frequency of transfer to fresh selective medium ***(21)***. However, it is important to note that conditions that result in an efficient transformation system for one genotype, do not always translate into an efficient system for other genotypes ***(18)***.

Our work required the development of an efficient transformation system for several different tomato lines for complementation analyses; testing of new vector systems; and studying value-added traits, promoter efficacy, and

functional genomics. Based on information from reports in the literature, we developed a standard tomato transformation protocol that has been routinely used by several research groups for transformation of various *L. esculentum* freshmarket cultivars including Moneymaker, Yellow Pear, Rio Grande, Momor, the processing line E6203, and Micro-Tom which was developed for the ornamental market. Average transformation efficiencies (the percent of explants that give rise to transformed plantlets) using this protocol ranged from 10 to 15%.

2. Materials

2.1. Tissue Culture Media

2.1.1. Stocks

1. 1X Murashige and Skoog (MS) basal salts mixture powder. If MS salts in powdered form are not available, MS major salt, minor salt and iron stocks can be prepared and stored at 4°C for several months. MS major salt stock (10X): 19 g/L of KNO_3, 16.5 g/L of NH_4NO_3, 4.4 g/L $CaCl_2 \cdot 2H_2O$, 3.7 g/L $MgSO_4 \cdot 7H_2O$, 1.7 g/L of KH_2PO_4. MS minor salt stock (100X): 0.62 g/L of H_3BO_3, 2.23 g/L $MnSO_4 \cdot 4H_2O$, 0.86 g/L of $ZnSO_4 \cdot 7H_2O$, 0.083 g/L of KI, 0.025 g/L of $Na_2MoO_4 \cdot 2H_2O$,1 mL of $CuSO_4 \cdot 5H_2O$ stock (2.5 mg/mL), 1 mL $CoCl_2 \cdot 6H_2O$ stock (2.5 mg/mL). MS iron stock (200X): 8.6 g/L of ethylenediaminetetraacetic acid (EDTA) ferric-sodium salt, light sensitive; store in brown bottle.
2. 0.4 mg/mL of thiamine-HCl, dissolve in H_2O; store at 4°C for up to 1 mo.
3. 0.5 mg/mL of pyridoxine-HCl, dissolve in H_2O; store at –20°C.
4. 0.5 mg/mL of nicotinic acid, dissolve in H_2O; store at –20°C.
5. 1 mg/mL of 2,4-dichlorophenoxyacetic acid (2,4-D), dissolve in H_2O, store at 4°C for up to 1 mo.
6. 1 mg/mL of kinetin, dissolve in a few drops of 1 *M* HCl; store at –20°C.
7. 2 mg/mL of glycine, dissolve in H_2O; store at –20°C.
8. 0.25 mg/mL of folic acid, dissolve in H_2O; store at –20°C.
9. 0.5 mg/mL of D-biotin, dissolve in H_2O, store at –20°C.
10. 1000X Nitsch vitamin stock: 2 g/L of glycine, 10 g/L of nicotinic acid, 0.5 g/L of pyridoxine HCl, 0.5 g/L of thiamine-HCl, 0.5 g/L of folic acid, 40 mg/L of D-biotin; adjust pH to 7.0 to clear solution, and store at –20°C.
11. 1 mg/mL of zeatin, dissolve in a few drops of 1 *M* HCl, filter-sterilize, store at –20°C.
12. Appropriate selective agent stock for vector, dissolve as necessary, filter sterilize, store at –20°C. (This protocol has been successfully used with kanamycin, hygromycin, and bialaphos as selective agents. All of these compounds are toxic and should be handled with appropriate care.)
13. 300 mg/L of timentin (a mixture of ticarcillin disodium and potassium clavulanate) or 500 mg/L of carbenicillin, dissolve in H_2O, filter sterilize, store at –20°C. These compounds are toxic and should be handled with appropriate care.

2.1.2. Media

1. 1/2 MSO medium: 1/2X MS salts; 100 mg/L of myoinositol, 2 mg/L of thiamine-HCl, 0.5 mg/L of pyridoxine-HCl, 0.5 mg/L of nicotinic acid, 1% and sucrose, 0.8% agar, pH 5.8. Autoclave and store at room temperature for up to 1 mo.
2. KC Biological MS (KCMS) medium: 1X MS salts, 100 mg/L of myoinositol, 1.3 mg/L of thiamine-HCl, 0.2 mg/L of 2,4-D, 200 mg/L of KH_2PO_4, 0.1 mg/L of kinetin, and 3% sucrose. For solid medium, add 0.52% Agargel, pH 5.5. Autoclave and store at room temperature for up to 1 mo.
3. Luria Bertani (LB) medium: 10 g/L of Bacto-tryptone, 5 g/L of yeast extract, 10 g/L of NaCl, 1.5% Bacto-agar Difco (BD, Franklin Lakes, NJ). Autoclave, cool to 55°C, add appropriate filter-sterilized selection agent, and store at 4°C (length of time depends on selection agent).
4. Yeast extract medium (YE): 400 mg/L of yeast extract, 10 g/L of mannitol, 100 mg/L of NaCl, 200 mg/L of $MgSO_4 \cdot 7H_2O$, 500 mg/L of KH_2PO_4. Autoclave and store at room temperature for up to 3 mo.
5. MS liquid medium: 1X MS salts, 100 mg/l myoinositol, 2 mg/L glycine, 0.5 mg/L of nicotinic acid, 0.5 mg/L of pyridoxine-HCl, 0.4 mg/L of thiamine-HCl, 0.25 mg/L of folic acid, 0.05 mg/L of D-biotin, 3% sucrose, pH 5.6, autoclave, store at room temperature for up to 3 mo.
6. 2Z medium: 1X MS salts, 100 mg/L of myoinositol, 1X Nitsch vitamins, 2% sucrose, 0.52% Agargel, pH 6.0. Autoclave; cool to 55°C; and add filter-sterilized stocks of vector-selective agent, timentin, or carbenicillin and 2 mg/L of zeatin. Store at room temperature for up to 1 wk.
7. 1Z medium: same composition as 2Z except zeatin is reduced to 1 mg/L. Store at room temperature for up to 1 wk.
8. Selective rooting medium: 1X MS salts, 1X Nitsch vitamins, 3% sucrose, 0.8% Bacto-agar, pH 6.0. Autoclave, cool to 55°C, and add filter-sterilized stocks of vector selective agent and timentin or carbenicillin. Store at room temperature for up to 1 mo.

2.2. Preparation of Plant Material

1. Seeds from *L. esculentum* line(s) of choice.
2. 20% Household bleach (1.05% sodium hypochlorite) plus 0.1% Tween-20.
3. Sterile distilled water.
4. Solid 1/2 MSO medium in Magenta (GA7) boxes.

2.3. Preculture

1. Tobacco suspension culture (approx 7 d after subculture) grown in liquid KCMS medium.
2. Solid KCMS medium in 100 × 15 mm petri dishes.
3. Sterile Whatman filter paper (7-cm circles).
4. Tomato seedlings (6–8 d old) obtained from **step 3.1.**
5. Sterile Petri dishes or paper towels.
6. Sterile distilled water.

2.4. Preparation of Agrobacterium tumefaciens

1. *A. tumefaciens* strain containing tumor-inducing (Ti) plasmid harbouring the gene of interest.
2. LB medium supplemented with appropriate selection agent in 100 × 15 mm Petri dishes.
3. YM liquid medium.
4. MS liquid medium.

2.5. Infection and Cocultivation

1. Precultured tomato explants prepared as described in **step 3.2.**
2. *A. tumefaciens* culture prepared as described in **step 3.3.**
3. Sterile Magenta boxes or other wide-mouthed containers.
4. Sterile paper towels.

2.6. Selective Plant Regeneration

1. Selective 2Z medium in 100 × 15 mm Petri dishes.
2. Selective 1Z medium in 100 × 20 mm Petri dishes and Magenta boxes.
3. Micropore filter tape (3M Corporation, St. Paul, MN).
4. Selective rooting medium in Magenta boxes.

2.7. Transfer to Soil

1. Four-inch pots.
2. Sterile potting mix.
3. Clear plastic bags or containers.

3. Methods

3.1. Preparation of Plant Material

1. Surface sterilize seeds in 20% household bleach for 10 min and rinse three times with sterile distilled water (*see* **Note 1**).
2. Culture on 1/2 MSO medium in Magenta boxes (25 seeds/box). Maintain at 24 ± 2°C, under a 16-h photoperiod (cool white fluorescent lights, 60–100 E/m^2/s for 6–8 d depending on the tomato line being used. Cotyledons should be expanded and seedlings should be used before first true leaves emerge.

3.2. Preculture

3.2.1. Preparation of Feeder Plates

1. One day prior to explant preparation, pipet approx 2 mL of a 7-d-old tobacco suspension culture onto solid KCMS medium in Petri dishes. (*See* **Note 2** for maintenance of the tobacco suspension.)
2. Seal plates with Parafilm and incubate overnight in the dark at 24°C ± 2°C.
3. Cover the suspension culture with a 7-cm circle of sterile Whatman filter paper.

3.2.2. Preparation of Explants

1. Remove seedlings to damp sterile paper towel or Petri dish containing sterile water and excise cotyledons (*see* **Note 3**).
2. Cut both ends of cotyledon to remove the tip and petiole. If cotyledon sections are longer than 1 cm, cut them in half (*see* **Note 4**).
3. Place cotyledon explants on feeder layer plates prepared the previous day as described in **Subheading 3.2.1.** At this stage as many as 80 explants can be cultured on a single plate; however, fewer should be used if contamination may be a problem.
4. Seal plates with Parafilm and culture for 1 d at 24 ± 2°C, under a 16-h photoperiod.

3.3. Preparation of A. tumefaciens

1. Streak *A. tumefaciens* strain onto fresh plate of LB medium containing the appropriate selection agent and incubate for 48 h at 28°C.
2. Transfer four well-formed colonies to a flask containing 50 mL of YM liquid medium supplemented with the appropriate selection agent and maintain in a shaking incubator at 28°C until an OD600 of 0.4–0.6 is reached (usually overnight).
3. Centrifuge the cells at 8000*g* for 10 min at 20°C.
4. Resuspend pellet in 50 mL of MS liquid medium.

3.4. Infection and Cocultivation

1. Transfer cotyledon explants prepared as described in **step 3.2** to 25 mL of prepared *Agrobacterium* suspension in a sterile Magenta box or similar wide-mouthed container and incubate for 5 min.
2. Remove bacterial suspension with a sterile pipet.
3. Blot explants on sterile paper towels and place with the adaxial sides down (that is, upside down) on the original feeder plates (*see* **Note 5**).
4. Maintain at 19–25°C in the dark for 48 h of cocultivation (*see* **Note 6**).

3.5. Selective Plant Regeneration

1. Transfer cotyledon explants to plates containing selective 2Z medium with the adaxial sides facing up (*see* **Note 7**). A total of 25 explants are cultured on each plate at this stage.
2. Seal plates with micropore tape and maintain at 24 ± 2°C under a 16-h photoperiod of cool white fluorescent lights (*see* **Note 8**).
3. After 3 wk, transfer the cultures to plates containing 1Z medium (*see* **Note 9**).
4. Transfer explants to fresh medium at 3-wk intervals (*see* **Note 10**). Discard explants that are completely brown or bleached and trim off dead tissue from regenerating explants. Approximately 10 explants are cultured on each plate at this stage—the exact number depends on the size of the callus and regenerating tissue.
5. When shoots begin to regenerate from the callus, transfer cultures to 1Z medium in Magenta boxes.

6. Excise shoots from callus when they are approx 2 cm tall and transfer to selective rooting medium in Magenta boxes (*see* **Note 11**).
7. Maintain plants at 24 ± 2°C under a 16-h photoperiod of cool white fluorescent lights.

3.6. Transfer to Soil

1. When the selected transgenic lines have well-formed root systems, they can be transferred to soil (*see* **Note 12**).
2. Remove a plant from its culture vessel and gently wash the medium from the roots. Use tepid water.
3. Transfer each plant to a 4-in. pot containing a sterile potting mix.
4. Cover each plant with a clear plastic container or a plastic bag secured to the pot to provide a humid environment (*see* **Note 13**). Transfer to a growth chamber or to a shaded area of a greenhouse. Do not place in direct sunlight (*see* **Note 14**).
5. After 1 wk, gradually lift the plastic container each day during the next week. If a plastic bag is used, cut a small hole each day during the course of the next week, then remove the bag.

3.7. Timetable for Tomato Transformation

This timetable is based on a 6-d germination period. Adjust the times to the germination period of your tomato seeds.

D 1. Sterilize seeds and transfer to 1/2 MSO medium (*see* **Subheading 3.1.**).
D 5. Streak *Agrobacterium* onto LB selective medium (*see* **Subheading 3.3.1.**).
D 6. Prepare feeder layer plates (*see* **Subheading 3.2.1.**).
D 7. Prepare cotyledon explants (*see* **Subheading 3.2.2.**).
Inoculate liquid overnight culture (*see* **Subheading 3.3.2.**).
D 8. Prepare *Agrobacterium* suspension for infection (*see* **Subheadings 3.3.3.** and **3.3.4.**).
Infect and cocultivate cotyledon explants (*see* **Subheading 3.4.**).
D 10. Transfer cotyledon explants to selective 2Z medium (*see* **Subheadings 3.5.1.** and **3.5.2.**).
D 31. Transfer cultures to 1Z medium (*see* **Subheading 3.5.3.**). Continue to transfer to fresh 1Z medium at 3-wk intervals (*see* **Subheading 3.5.4.**).

4. Notes

1. If contamination is a problem, the seeds can be soaked for 2 min in 70% ethanol before the bleach treatment.
2. The tobacco suspension culture (NT1) is maintained in liquid KCMS medium on a rotary shaker and is subcultured weekly by adding 2 mL of the old culture to 48 mL of fresh medium.
3. Remove only as many seedlings as can be prepared in a few minutes, as they wilt quickly.

4. Hypocotyls may also be used but they regenerate more slowly and tend to produce more nontransformed shoots.
5. Explant orientation at this step is only for convenience.
6. Culture at lower temperatures has been associated with higher transformation efficiencies.
7. Explant orientation at this step has a significant effect on transformation efficiency. Explants placed adaxial side up curl into the medium, make better contact with it, and produce more transformed shoots.
8. Micropore tape gives higher transformation efficiency than Parafilm.
9. Zeatin level is reduced at this step to conserve resources. The reduction does not have a negative effect on transformation efficiency.
10. Transfer is necessary every 3 wk as plates sealed with micropore tape dry out quickly. More frequent transfers do not increase transformation rate.
11. Do not transfer shoots without meristems because they will not develop meristems in rooting medium.
12. To ensure that the plants are free of *Agrobacterium* before transfer to soil, shoots can be rooted twice on medium containing timentin or carbenicillin and then once on medium lacking this antibiotic. Plantlets that show *Agrobacterium* contamination at this stage should be destroyed by autoclaving. Alternatively, a PCR assay with *Agrobacterium*-specific primers can be done to ensure that plants are not contaminated.
13. Cover each plant with either the plastic container or a bag immediately after transfer to soil. The plants wilt quickly after removal from the culture containers. For plastic containers, Magenta boxes can be used or even clear plastic bottles that have the top of the bottle removed.
14. If placed in direct sunlight, heat will build up under the plastic containers or bags and kill the plants. If a growth chamber or shaded area of a greenhouse is not available, then maintenance in a lab setting under lights will be sufficient for the first 2 wk. After that period, they can be moved to a greenhouse.

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