Transformation of tomato with *Agrobacterium tumefaciens*

SHEILA McCORMICK  
*Plant Gene Expression Center, USDA/ARS–UC-Berkeley, 800 Buchanan St., Albany, CA 94710 USA*

Introduction

*Agrobacterium*-mediated transformation and regeneration of a variety of tomato (*Lycopersicon esculentum*) cultivars was first reported by McCormick *et al.* [12]; other procedures for *L. esculentum* transformation include those of Fillatti *et al.* [5] and Bird *et al.* [2]. Chyi *et al.* [4] published a protocol for transformation of *L. esculentum* cv. VF36 × *L. pennellii* (LA716) interspecific hybrids, and procedures for transformation of a derivative of an interspecific hybrid between *L. esculentum* and *L. peruvianum* [9, 11] are available. Since 1986, numerous groups have used these procedures or modifications thereof to obtain transgenic tomatoes [e.g., 3, 7, 14, 15] for analysis of gene expression. However, tomato is still considered more difficult to transform than species such as *Petunia hybrida* and *Nicotiana tabacum*, and can show widely different success rates, possibly depending on cultivar, *Agrobacterium* strain, antibiotic selection, and/or the personnel performing experiments. This paper describes a detailed protocol with trouble-shooting comments that should serve as a basis for initiating a tomato transformation program, or for improving an existing program.
1. Sterilize dry seeds in 20% household bleach + 0.1% Tween-20 for 15 minutes, followed by 3 or more rinses in sterile H₂O. Alternatively, a 5 minute treatment with 50% household bleach, followed by rinses with sterile water can be used with seeds directly extracted from fruit.

2. Germinate the sterilized seeds on 1/2 × MS0 in sterile magenta boxes (obtainable from Sigma). Cover bottom of box with a dense monolayer of seeds (100 or so); grow at 26 °C under 16L/8D light conditions for 10–14 days.

3. Cotyledons can be used for transformation when there are no or minimal true leaves present on seedlings. The tops of the seedlings (approx. the top half of hypocotyl and attached cotyledons) are cut off in batches and floated in MS0 liquid for cutting. Cutting the cotyledon explants is conveniently done in a 150 × 15 mm Petri dish. Approximately one third of the seedlings in a magenta box are removed and cut at a time; this ensures that the cotyledons don’t dry out while waiting to be cut and placed on medium. Cotyledons are cut near the proximal (wide) end (an additional cut surface near the distal end can be made, but one is usually sufficient). Attempts should be made to cut the cotyledons when they are submerged in the MS0. The cotyledons are placed upside down on 100 × 15 mm Petri dishes containing D1 medium. 500 ml of medium should yield 20 plates. The cotyledons are placed very densely (sides touching) on the plate (50–75 cotyledons per plate). Alternatively, cotyledons are placed upside down on MS0 plates (with hormones appropriate for callus formation) with a 1 ml layer of *Nicotiana tabacum* feeder cells under a 3MM filter paper disc, cut to fit precisely, as described in McCormick *et al.* [12].

4. 5 ml of a 20-fold diluted *Agrobacterium* 2 day ‘overnight’ culture is poured over the cotyledons on the surface of the plate, incubated for 1–2 hrs, then the excess sucked off with a pipette (usually you can remove 3–4 ml of solution from the plate surface). The plates can be swirled occasionally during the 1–2 hr incubation to ensure that the cut surfaces get wet. The cotyledons can be straightened out (but still upside down) so they are not clumped to one side on the plates. Then the plates are taped shut with two pieces of time tape and incubated in a growth room (26 °C, 16L/8D) for 2 days. For leaf transformation, or for cotyledons on feeder plates, it is possible to treat with *Agrobacterium* as above; alternatively, the cotyledons or leaf pieces can be swirled in a dish until the edges are wet, and then blotted dry and placed on the feeders for co-cultivation, as described in McCormick *et al.* [12].

5. After two days co-cultivation, the cotyledons are transferred to deep plates (100 × 20 or 25 mm) of D1 medium + 500 µg/ml carbenicillin + 100 µg/ml kanamycin. Ten to twelve plates can be poured from 500 ml of medium. The cotyledons don’t need to be blotted dry. Cotyledons should be placed upside down on the selection plates, the plates sealed with tape, and returned to the growth room for incubation. Callus, green bumps, or shoots usually will be seen at the cut edges within 10 days, and certainly after 3 weeks incubation, if the transformation was successful.
6. Cotyledons are transferred at 3 weeks to D2 medium (deep plates) + 500 µg/ml carbenicillin + 100 µg/ml kanamycin, for further shoot organogenesis. At the first transfer to D2 plates, the entire explant should be transferred; at subsequent 3 week transfers, the callus/shoot should be excised away from the dying cotyledon.

7. After another three weeks, often shoots with true meristems will have arisen, and can be cut off cleanly from the callus/cotyledon and transferred to rooting medium (MS0 + carbenicillin + kanamycin, but no hormones). Shoots will usually root within 7–10 days.

8. Shoots can then be transferred into very wet soil in a 2 inch pot within a magenta box, with the lid tightly closed. Any residual agar should be rinsed from the roots before transplanting. These magenta boxes are incubated under low light conditions (1200 ft-c) (incandescent + fluorescent) and the lid of the magenta box slowly tilted off over a period of 5 days. The transformants are left within the magenta box (without the lid) until they are a reasonable size. They are then removed from the magenta box, and eventually potted up and transferred to the greenhouse.

9. R1 seed is scored for kanamycin resistance by sterilization and germination on 1/2 MS0 + 100 mg/l kanamycin in magenta boxes. Resistant seedlings will be taller, will have branched roots and reduced or no anthocyanin pigmentation in the hypocotyls. Sensitive seedlings will germinate but will be short and stunted, will have stubby unbranched roots, and often have enhanced anthocyanin pigmentation in the hypocotyls. Scoring is most reliable at approximately 2 weeks after seed are set out, but can be done up to a month after germination.

Notes

1. The above protocol was optimized for the cultivar VF36. VF36 seeds are not commercially available, and thus have to be bulked up by the researcher. In addition to the cultivars cited in McCormick et al. [12], other cultivars that give a reasonable frequency of transformants with this protocol are New Yorker and the interspecific cross L. esculentum cv. VF36 X L. pennellii (LA716). Cultivars that did not respond favorably to this protocol include Vendor and VFNT cherry; the protocol of Fillatti et al. [5] reportedly is successful with VFNT cherry [15]. Before any new cultivar is used for transformation, it is strongly recommended that an optimized regeneration protocol be developed, based on protocols from existing literature or by empirical tests of different hormone grids.

2. The cotyledons should be dark green and the seedlings compact, not pale green and spindly. This can be assured by dense platings of the seeds, 50 ml volumes of 1/2 MS0 in the magenta boxes for germination, and use within 10–14 days after seed germination. Some cultivars or experiments will show anthocyanin pigmentation in the hypocotyls, or on the abaxial surface of the cotyledons during culture (a probable stress response). However, there is no particular correlation between successful transformation and any of these features. Hypocotyls can also be used for transformation, although they are not as efficient in generating transgenic shoots, and the shoots take longer to develop. For these reasons, hypocotyls are not worth the extra effort, unless the plant material is limiting.

Leaf pieces from greenhouse-grown 5–6 weeks old plants (optimally), or young healthy leaves from older plants can be used if required, although success with leaf transformation is more of an 'art' than with cotyledons. For certain experimental protocols (such as re-transformation of transformants) leaf transformation is more expedient than waiting for F1 seed from the transformants. Batches of medium can be made and autoclaved in 500 ml aliquots and stored for months at room
temperature, then melted in the microwave and hormones and antibiotics added before pouring plates. Carbenicillin (250 mg/ml), kanamycin (50 mg/ml) and zeatin (5 mg/ml) are stored as stocks at -20°C, melted and added to stored medium just before pouring plates. Poured plates with kan/carb/zeatin can be stored for at least 1–2 months at 4°C.

3. Cotyledons can be pre-callused, or pre-incubated for 1–2 days before addition of Agrobacterium. Select for transformation only the cotyledons which swell in size. This is extra work, but may be worth trying if the cut edges of the cotyledons are turning brown. Usually 2 plates per construct per transformation date is sufficient to obtain at least 10–20 independent transformants, although these transformants might be transferred to rooting medium and to soil over a period of several weeks. A typical transformation (performed on 6/13/89) with two plates of cotyledons yielded 26 independent transformants that were transferred to soil during the period of 8/23/89 to 9/25/89.

For various reasons (receptivity of cotyledons, Agrobacterium overnight not at right cell density, ‘bad’ day) experiments occasionally do not give a good yield of positives within the first three week incubation. It is more effective to do multiple 2 plate transformations on different days, than to do a large scale transformation on a particular day (if 2 plates give unsatisfactory efficiencies, doing 8 plates with the same material will not help matters).

For cotyledon transformations, acetylsyringone (final concentration of 375 μM) can substitute for N. tabacum feeder layers, and saves two days time. Feeder layers are recommended for leaf transformations, although acetylsyringone can be used. Feeders are prepared and used as in McCormick et al. [12]. A N. tabacum suspension culture is grown in liquid MSO medium containing 2 mg/l p-chlorophenoxyacetic acid. The suspension is subcultured weekly.

4. Although it seems reasonable to place the cotyledons on the plates right-side up, so that the cut edge is in contact with the medium, experience suggests that better regeneration is attained (and few to no escapes obtained) if the cotyledons are placed upside down for selection/regeneration. Parafilm can also be used to seal plates, but more condensation occurs on the plate lids. Time tape closure is fine if a reasonably sterile growth room is used to incubate the plates. However, if there is a problem with fungal contamination, use parafilm.

Agrobacterium (2 days) overnights are started from glycerol cultures stored at -80°C, and grown in 5 ml cultures in LB, with no selection in 250 ml Erlenmeyer flasks at 29°C, shaking at 250 rpm. These are diluted 1:20 into liquid MSO, and acetylsyringone added (final concentration of 375 μM).

If problems with Agrobacterium overun occur, or if no transformants are obtained, a more careful study of bacterial titer might be warranted; however, it is usually not necessary. Acetylsyringone is stored in EtOH at -20°C, stock 0.0148M. (Conveniently, then 25 μl is added per ml of diluted Agrobacterium to give a final concentration of 375 μM). If the Agrobacterium culture has to be grown under selection, then the culture should be spun down and resuspended in 5 ml LB without antibiotics, and treated as above.

This protocol has been successful with both cointegrate [6, 16] and binary vectors [1, 10], although problems with a particular binary construct (pMON505) were noted [12].

5. Typical experiments should give at least 50–60% of the cotyledons regenerating callus, green bump structures, or shoots within the first 3 weeks, which should result in 10–20 independent transformants in soil within 9 weeks. Different cultivars and/or Agrobacterium strain combinations may take longer or shorter to achieve these numbers of transformants. If there are no visible calluses or green bumps during the first three week incubation, it is not worth transferring these cotyledons to another three weeks incubation on D1 selection plates; it is better to attempt a new transformation. Other selectable markers that can be used with tomato include hygromycin [13] and gentamycin [8]. Some Agrobacterium strains carry resistance to carbenicillin; claraon can be used to control the bacteria, but the cotyledons may have to be transferred at more frequent intervals than every three weeks (because claraon is light-sensitive). In this case, transfers should be made so that the cotyledons are exposed to 1 mg/l zeatin for three weeks, and then transferred to 0.1 mg/l zeatin plates.

6. A convenient method of keeping track of independent events is to assign a letter (a, b, c, ... z, a², b², ...) to each cotyledon that is to be transferred. Occasionally, shoots that appear abnormal, or shoots with unusual leaf shapes will arise from the...
cut edges of the cotyledons — if deemed necessary (because of lack of an abundance of normal
shoots), these abnormal shoots can be transferred several times and usually will eventually ‘grow
out’ of the problem and a normal meristem will arise.
It is usually not worth the effort to do more than 3 serial transfers onto D2 selection plates, because
you reach a point of diminishing returns. If something hasn’t regenerated normal shoots by then,
it probably won’t. However, if a particular experiment looked promising at the 3 week point, but
it is difficult to obtain shoots, it might be worthwhile to try a different hormone regime to stimulate
shooting; for example, maintaining selection, but switching from 0.1 mg zeatin/l plates to 1 mg/l
benzyladenine (BA) + 0.1 mg/l indole acetic acid (IAA) for a 3 weeks interval.
7. Any shoots that arise during the second or subsequent transfers (D2 + selection plates) should
be transferred to rooting medium as soon as they look big enough; it is not necessary to only do
transfers at three week intervals if something looks ready to go to the next step.
Plantlets should have 2 to 3 good roots and an obvious meristem before transfer to soil. Some
transgenic plants may not root (for physiological reasons) on selection plates, but remain green and
healthy, and continue to grow. Such plants can be dipped in Rootone and potted into soil as above;
they are often bona fide transformants.
During the shoot initiation and shoot elongation steps (D1 and D2 media) it is important to maintain
kanamycin selection at 100 μg/ml, rather than reducing the level and hoping thereby to obtain more
shoots. Shoots that arise at 25–50 μg/ml kanamycin are likely to be escapes.
8. Plants should be acclimated to larger pots and allowed to become sturdy plants under the 1200 ftc
light conditions before transfer to the harsher conditions of the greenhouse.
9. Only one obvious, probable, somaclonal mutation (e.g. chlorophyll defects) was noticed in the
process of screening R1 seed from over 100 independent transformants that were generated using
this transformation procedure (McCormick, unpublished). Yoder et al. [15] reported a mutation
rate of over 20% in VF36 transformants — possibly, differences in the transformation regime can
account for this difference.

Media

- **MSO:**
  - 4.3 g/l MS salts (Gibco)
  - 3% (w/v) sucrose
  - 1X Gamborg’s B5 vitamins (Gibco or Sigma).
  - 0.8% (w/v) agar, for solid medium
    - adjust pH to 5.8 with 1M KOH

- **1/2xMSO:**
  - 2.3 g/l MS salts (Gibco)
  - 3% (w/v) sucrose
  - 1X Gamborg’s B5 vitamins (Gibco or Sigma)
  - 0.8% (w/v) agar
    - adjust pH to 5.8 with 1M KOH

- **D1 medium:**
  - 4.3 g/l MS salts (Gibco)
  - 3% (w/v) glucose
  - 1X Gamborg’s B5 vitamins (Gibco or Sigma)
  - 1 mg/l zeatin
  - 0.8% (w/v) agar
    - adjust pH to 5.8 with 1M KOH

PTCM-B6/6
- **D2 medium:**
  - 4.3 g/l MS salts (Gibco)
  - 3% (w/v) glucose
  - 1× Gamborg’s B5 vitamins (Gibco or Sigma)
  - 0.1 mg/l zeatin
  - 0.8% (w/v) agar
    - adjust pH to 5.8 with 1M KOH

- **LB (Luria–Bertani) medium:**
  - 10 g/l bacto-trypthone
  - 5 g/l bacto-yeast extract
  - 10 g/l NaCl
    - adjust pH to 7.5 with 1M NaOH
References