

Cotton Tissue Culture and *Agrobacterium* mediated Plant Transformation

(Chapman Laboratory Protocols, Drs. Shanmukh Salimath and Purnima Neogi)

This protocol is intended for those reasonably familiar with plant tissue culture technology and its applications and with basic laboratory experience (handling chemicals, preparation of solutions, microbiology and aseptic culture techniques, etc.).

For additional information, see references at end of protocol or contact:

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I. Seed Germination, Callus induction and Somatic Embryogenic Cell Line generation

A. Delinting of Cotton seeds (carry out this step in acid/organics fume hood)

Place about 25 fuzzy cotton seeds in a conical flask/beaker. Add concentrated sulfuric acid (H_2SO_4) so as to cover the seeds. Stir slowly with long glass rod for 30 - 60 seconds. Drain off sulfuric acid into an empty beaker and add the seeds, along with remnants of acid, into a 500 mL beaker with distilled H_2O . Change H_2O few times. Retain seeds in the glass jar with the help of strainer/miracloth. Put a rubber band over miracloth around the mouth of the beaker and wash the seeds under running tap H_2O for about 2 hours.

B. Surface sterilization of cotton seeds. The entire procedure, here onwards, has to be done under plant tissue culture hood in order to avoid fungal/microbial contamination.

Transfer the seeds (from A above) in to a clean sterile flask or beaker with sterile MilliQ ultrapure H_2O . Rinse well, by shaking vigorously, 2-3 times with sterile MilliQ ultrapure H_2O .

Transfer the seeds to 100mL of 70% ethanol + 2 drops of Tween 20 for 1 min. Rinse again with sterile MilliQ ultrapure H_2O (2 times - 2 min each).

Next surface sterilize the seeds by treating with 10% bleach + 2 drops of Tween 20 for 1-2 min and then wash 3-5 times in sterile MilliQ ultrapure water.

Surface sterilized seeds can be soaked overnight, under the hood, in sterile MilliQ ultrapure water for softening of seed coat. However, this is not absolutely necessary.

Place surface sterilized seeds on MSM [Murashige and Skoog, MS salts + 3% Maltose + MS vitamins (Gamborg's vitamins) (pH 5.7) + 0.8% Phytigel] plates.

Keep the plates in a growth chamber at 28°C (16h light/8h dark) for 3-5 days.

Transfer germinated cotton seeds to 10-15mL MSM liquid media, in a deep dish culture plate, to aid in cotyledon expansion for 2-5 days. When cotyledons have fully expanded and look fleshy and green start leaf *explant* culture.

C. Tissue culture

With the help of a sharp blade/scalpel cut the cotyledonary leaves into small segments (15 mm²) and place them on deep dish tissue culture plates with MSMK media for 2-3 weeks [(MS + 3% Maltose + vitamins + 1.9 gm/L KNO₃ (adjusted to pH 5.7 with 1N NaOH) + 0.8% Phytigel].

Tissue culture plates have to be kept under a 16 h (70 μmol m⁻² s⁻¹) photoperiod at 28°C.

Move the sub-cultures to fresh media plates every 3-4 weeks until callus production.

D. Generation of Somatic Embryogenic Cell Lines (SECL)

Identify pale green/yellowish rapidly growing calli in the tissue culture plates. Collect somatic embryogenic cells (SECs) from callus cultures and initiate generation of SECLs in MSM liquid medium (3-4mL per well) in a 12 well tissue culture plate on a gyratory shaker (110rpm, 16 hr light/8hr dark) at 28°C- (3-4 weeks).

Multiply SECs in MSM liquid medium (3 - 4 ml) in 12 well tissue culture plates for use in transformation experiments - (3-6 weeks).

II. *Agrobacterium* – mediated cotton transformation:

A. Agro – SECL co-cultivation and transformation

Use actively growing cotton Somatic Embryogenic Cell lines (SECL), maintained at 28°C for *Agrobacterium* mediated cotton transformation.

Culture *Agrobacterium tumefaciens* strain LBA4404 cells (containing gene of interest in binary vector) at 28°C for 36-48 hours in 5mL LB medium with streptomycin (100μg/mL) and kanamycin (50μg/mL) (or appropriate antibiotic for bacterial selection of binary vector).

Using 5 mL culture as inoculum to start a large scale culture of Agro in fresh (100 to 200mL) LB medium [with streptomycin (100μg/mL) and kanamycin (50μg/mL)] for 36 to 48 hrs.

After two overnights, collect large scale Agro cultures into 50mL sterile blue cap tubes and spin on table top centrifuge at 25°C, 3000rpm for 15 to 30mins. Agro cells form a compact pellet at the bottom. Decant the media.

Re-suspend the Agro cell pellets in 10-15mL MSM liquid medium containing Acetosyringone (100μM final concentration) to obtain an A₆₀₀ of 0.3-0.4.

Harvest Embryogenic Cell Lines and flood them with MSM re-suspended Agro cells in a sterile deep dish petri plate.

The embryogenic calli (SECLs) are infected with bacterial suspension in a sterile deep dish tissue culture plate with intermittent vigorous shaking for 5 - 10 min.

Remove excess media with sterile 10 mL pipette. With the help of a clean, sterile spatula transfer the SECS to MSM layered with a sterile filter paper (disc) and topped with MSM +100µM acetosyringone liquid media.

SECs and *Agrobacterium* cells are co-cultured in the dark @ 25°C for 48 hours.

Transfer SECs, in small (2-5mm) tissue mounds, to MSMK (MSM + KNO₃) solidified media containing carbenicillin (400µg/mL) and a plant-selection antibiotic (e.g. kanamycin 50 µg/mL or hygromycin 15 to 25µg/mL etc.).

Keep the plates in a growth chamber at 28⁰C (16h light/8h dark) to select for developing transgenic embryos.

Collect differentiating embryos onto fresh MSMK media with carbenicillin + selection antibiotic every 3-4 weeks. When the embryos are differentiated into young plantlets transfer them to MSM + selection antibiotic in magenta boxes.

B. Moving plants from Lab to the Greenhouse

When plantlets are slightly larger (approximately 2-3 cm), transfer them to autoclaved soil media containing Rotone (root inducing hormone) in 4X4 inch pots. Cover the plants with transparent plastic bags for the first 4-5 days. Establish and acclimatize the plantlets to soil media in Plant Growth Chamber for 7-10 days at 28⁰C (16h light/8h dark)

When plants are at 2-4 leaf stage move them to the greenhouse for seed production.

Following these methods we are able to move primary (T₀) transgenics to the greenhouse as early as 3-4 months and harvest the T₁ seeds within 6-8 months of co-cultivation.

Maltose-containing Murashige and Skoog medium (MSM medium) – 1 Liter volume (suggested recipe)

100mL - 10X MS Basal Salt Macronutrient solution (Sigma)

100mL - 10X MS Basal Salt Micronutrient solution (Sigma)

1mL -1000X MS Vitamin solution (Sigma)

30gms – D-Maltose (Sigma)

Dissolve in MilliQ H₂O, adjust pH 5.7 with 1N NaOH and then bring the final volume to 1 liter. Add 8 gms Phytigel (for solid media only). Autoclave the media for 20 Minutes under liquid cycle. When cooled down (~ 60⁰C) add antibiotics (carbanecillin, kanamycin etc.) and pour the plates. Pouring plates etc. needs to be strictly done under the plant culture hood.

*If making MSMK media add 1.9gms of potassium nitrate (KNO₃) per liter before autoclaving.

References

1. Leelavathi et al (2004). Plant Cell Rep 22: 465-470.
2. Rathore et al (2006). Cotton. In Methods in Molecular Biology, Vol 343: *Agrobacterium* Protocols, 2nd ed., Vol I I. pp. 267-279. (Edtd. By Kan Wang, Humana Press Inc. Totowa, NJ.
3. Wu et al (2008) Plant Mol Rep 26: 174-185.
4. Salimath et al., (2010) Proceedings of the National Cotton Council Beltwide Cotton Conferences, New Orleans, Louisiana (<http://www.cotton.org/beltwide/proceedings>).