TRANSFORMATION OF ARABIDOPSIS THALIANA USING VALVEKENS'S PROTOCOL: IMPROVED ROOT HARVEST
(This protocol, updated on 3 July 1991, was made available via the electronic Arabidopsis bulletin board.)

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* What follows is a detailed protocol which I follow when transforming Arabidopsis. It differs from the Valvekens (Valvekens, D., Van Montagu, M. & Van Lijsebettens, M. 1988. Agrobacterium tumefaciens- mediated transformation of Arabidopsis thaliana root explants by using kanamycin selection. Proc. Natl. Acad. Sci. USA 85, 5536-5540) protocol only in a few details, most of which were adopted for convenience rather than for an improvement in efficiency. The one change that I do feel improves efficiency is the omission of MES from all media. Other items to note. Not all commercial MS salts contain the same components. Gibco MS salts differ from Hazelton, for example, and may not work quite as well (I've not done a controlled experiment on that one). The choice of agar/agarose used in your plates can make a dramatic difference, especially when choosing an appropriate antibiotic concentration for selection. In a controlled experiment using seedlings from bona-fide transformants, I found that the seedlings were 10 times more sensitive to kanamycin on BRL agarose compared to N.C. Biologicals T.C. agar. Many of you have asked about ecotype differences. I have had the best luck with ecotype No-0, but also had very good results with Col-0 La-er, and Po-1. Ws-0 did not work well for me, but still gave me a few verified transformants. I have not tried any other ecotypes.

1. MEDIA
   (i) GERMINATION MEDIUM (GM)
   0.5 x Murashige & Skoog salt mixture (Hazelton Biologics)
   1 % sucrose
   pH 5.7, adjusted with 1 N KOH before autoclaving
   0.8% tissue culture grade agar (e.g., Carolina Biologicals T.C. agar; 0.5% BRL agarose also works very well)
   1 ml l-1 1000x Gamborg's B-5 vitamins (added after autoclaving)
   1000x B-5 vitamins 10 ml l-1
   myo-inositol 1 g
   Thiamine HCl 0.1 g
   Nicotinic acid 10 mg
   Pyridoxine HCl 10 mg

   (ii) 0.5/0.05 CALLUS-INDUCING MEDIUM (CIM)
   1 x Gamborg's B5 medium with sucrose (Gibco)
   pH 5.5, adjusted with 1 N KOH before autoclaving
   0.8% agar (for solid media)
   0.5 mg l-1 2,4-D
   0.05 mg l-1 kinetin

   (iii) 0.5/0.05 WITH CARBENICILLIN
   As 0.5/0.05, but supplemented with
   500 mg l-1 carbenicillin

   (iv) 0.15/5.0 SHOOT INDUCING MEDIUM (SIM)
1 x Gamborg's B5 medium with sucrose (Gibco)pH 5.5, adjusted with 1N KOH before autoclaving
0.8% agar
5.0 mg/l N6-(2-isopentenyl)adenine (2ip)
0.15 mg/l indole-3-acetic acid (IAA)

(v) 0.15/5.0 WITH HYGROMYCIN OR KANAMYCIN
As 0.15/5, but supplemented with
500 mg/l carbenicilllin
20 mg/l hygromycin or 50 mg/l kanamycin

(vi) SHOOT ELONGATION MEDIUM (EM)
1 x Murashige & Skoog salt mixture (Hazelton Biologics)
10 g/l sucrose
pH 5.8, adjusted with 1 N KOH
0.5% agarose (seed set seems to be better on agarose)
1 mg/l Indole Butyric Acid (IBA), made fresh every time
1 ml 1000x Gamborg's B-5 vitamins
500 mg/l carbenicilllin

* All media should be poured into plates in the rear half of a laminar flow hood and allowed to dry completely before putting on the lids (30 min in Berkeley, longer in Indiana). This step helps prevent bacterial overgrowth and encourages seed set.

* All hormones, antibiotics, and vitamins are added after autoclaving. Sigma now sells "Plant Cell Culture Grade" reagents (sucrose, vitamins, hormones, etc.), which are worth trying if problems regenerating plants without selection are encountered. For convenience, media are made up ahead of time as a 2 x stock without agar, antibiotics, or hormones, typically 200 ml in 500 ml bottles. An equal volume of sterile 2 x water agar (1.6%) is melted in a microwave and added to the bottle containing the 2 x medium and then hormones, etc. are added. Hormones and antibiotics are made up as 1000 x stocks in DMSO and distilled water, respectively, and filter sterilised. These can be stored at -20°C for several weeks, except for IBA, which should be made up freshly. GM is poured into Falcon 1013 plates (150 x 25 mm). SIM and CIM are poured into Labtek 4026 plates (100 x 25 mm) and EM is poured into Magenta GA7 boxes. Plates are sealed with a gas permeable tape (e.g., 3M Filter Tape, Carolina Biologicals Cat. No. 19-9708, or 3M Transpore Surgical Tape available in most drug stores).

2. SEED STERILISATION
   (i) Submerge seed in 70% ethanol for 5 min
   (ii) Rinse seed 2 x in sterile distilled water
   (iii) Submerge seed in 2.6% sodium hypochlorite (0.5 x standard laundry bleach)/0.05% Tween for 10 min, agitating regularly
   (iv) Rinse seed 4 x in sterile distilled water. (v) After last wash, keep seeds in ~0.5 ml water; using a sterile glass pasteur pipette, take up seed and spread onto GM plates, about 100 per plate
   (iv) Place seeded GM plates at 40°C for 48 h to break dormancy.

   * I suggest trying Luca Comai's trick of lining the seeds up along the top third of the plate and then placing the plate vertically in the growth chamber.

3. Growth of seedlings
   (i) Incubate cold-treated seeds on GM in a clean controlled environment
(24°C; 15 h light/9 h dark; 70-100 uEm-2s-1 light intensity). Seeds should germinate within 3 d. Roots are usable for transformation after 20 d and are good until about 35 d.

4. Transformation of root explants
   * All manipulations are done in a sterile hood.

   (i) Pull plantlets (20-35 day old plants) gently out of the GM using a forceps.
   (ii) Put plantlet in a sterile empty petri dish. Cut off root system from rest of plant so that no green parts remain attached to the root. Immediately place intact root system on CIM (no antibiotics). Spread flat so that all rootlets are in contact with plate. Place several root systems on a single CIM plate.
   (iii) Incubate in growth chamber (same settings as for growth of plants above) for 3 d.
   (iv) After the 3 d "pretreatment", remove intact root systems from CIM plates and place in 50 ml Corning sterile polypropylene tube containing 10 ml of liquid CIM.
   (v) Add 0.5 ml of 24-48 h Agrobacterium culture and vortex gently. The Agrobacterium is grown in L-broth supplemented with appropriate antibiotics (50 mg/l kanamycin, 25 mg/l chloramphenicol, 50 mg/l spectinomycin, and 30 mg/l streptomycin for strain ASE with a pMON based vector). The culture should be saturated at the time of adding to the roots.
   (vi) Let sit for about 2 min to let roots settle, then decant liquid. Remove clump of roots with forceps and place in a sterile petri dish containing several Whatman filter papers to blot up excess liquid.
   (vii) Transfer intact roots to CIM plates.
   (viii) Cut root systems into 0.5 cm segments using sterile scissors and spread out "clumps" of root pieces, but keep root pieces within a clump together. * One root explant consists of several 0.5 cm root segments and can be considered as a cutting of about 0.5 cm through an entire root system.
   (ix) Incubate infected root explants for 48 h, by which time explants should be overgrown by a thin Agrobacterium slime.
   (x) Wash off Agrobacterium by transferring explants to Corning 50 ml tube containing 25 ml 1 x B5 medium (no hormones) supplemented with 500 mg/l carbenicillin. Vortex gently and let root pieces settle. Decant liquid slowly into the lid of a sterile petri dish keeping as many of the root pieces in the tube as possible. When only a couple of ml of liquid remains in the tube, dump liquid and roots onto Whatman filter disks placed in the bottom of the petri dish to absorb the rest of the liquid. Use forceps to transfer any escaped root pieces in the lid to the filters in the dish. Blot the top of the root pieces with another Whatman filter disk.
   (xi) Transfer root pieces to SIM plates containing carbenicillin (500 mg/l) and the appropriate selective antibiotic (typically 50 mg/l kanamycin sulfate (Sigma) or 20 mg/l Hygromycin B (Boehringer)). Reassemble root explants by clumping together root pieces.
   (xii) Incubate in growth chamber for 2-3 weeks. Tiny green calli covered with root hairs should start appearing in about 6 d. On kanamycin the rest of the root explant remains white. On hygromycin the explant turns yellow then brown. The green calli should continue to enlarge. Development of shoots can be expedited by transferring to fresh media every 7 d.
   (xiii) During the third week, cut the green calli away from the dead explant material and transfer to a fresh SIM plate containing the same
antibiotics. Shoots should soon start appearing. Onset of shooting is highly dependent on the Arabidopsis ecotype used. We use No-0, which shoots very rapidly. Columbia is slower to shoot, but will shoot if explants are transferred to fresh medium every 7 d. Landsberg (La-0) and Po-1 shoot well.

(xiv) After shoots form a small rosette, transfer whole callus and rosette to a Magenta jar containing EM (note that carbenicillin is present in EM, but the other antibiotics are omitted at this point). Keep lid on tight initially. The plant should bolt within 2-3 weeks, at which time the explants should be transferred to a fresh EM Majenta jar (one plant per jar) that has a ~1 inch hole cut in the lid (a #14 cork borer works nicely for this modification). The hole is plugged with a foam stopper. This allows the humidity to drop to a level that allows seed to set, while still maintaining sterility. Plants must be transferred to fresh jars every 7 d as the agarose will dry out. Plenty of seed should set in these assemblies, but once roots form (not all explants will form roots) they can be transferred to soil. Seed production is better in plants growing in soil, but I do not usually do this as seeds can be bulked up in the next generation.

(xv) To put plants in soil, transfer them to 2 inch pots in Majenta jars that have been autoclaved with soil in pot and saturated with water containing a fertiliser (e.g., 0.5 x MS salts or 0.25 x Rapid-Gro Plant Food 23-19-17). After putting plants in the soil, put lid on tightly for 3 d, then slowly crack open to allow gradual drying. After 5 d the lid can be completely removed. Do not keep soil soggy as this encourages rotting. Just keep slightly damp.

5. COMMENTS

* If carbenicillin cannot be used to counterelect the Agrobacterium strain used, vancomycin may be substituted. However, vancomycin is not as effective and must be taken to wash roots thoroughly after the infection (i.e., do two or three successive washes in SIM containing 1000 mg/l vancomycin and blot well before placing back on plates containing 750 mg/l vancomycin). In addition, explants should be transferred to fresh plates every 7 d at a minimum.

* I have heard that other people have had difficulty with the hygromycin selection using this protocol, but I have not. I suspect that it is the promoter driving Hyg that makes the difference. I use a 35S Hyg gene from the vector pMON408.