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Agrobacterium-mediated transformation and regeneration of cotton

Thea A. Wilkins[1*], Rajiv Mishra[1] and Norma L. Trolinder[2]
[1]Department of Agronomy & Range Science, University of California, One Shields Ave., Davis, CA 95616-8515
*e-mail: tawilkins@ucdavis.edu. [2]Department of Crop and Soil Science, Texas Tech University, USA.

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Abstract

The development of transgenic cotton requires an efficient means for the transformation and regeneration of fertile plants. Despite the commercial success of genetically modified cotton, the transformation and regeneration of cotton is still challenging relative to other crop species. The efficiency of somatic embryogenesis and somaclonal variation are among the most often cited problems encountered in the regeneration of cotton. In the last decade, numerous innovations have been incorporated to improve the recovery and germination of high quality somatic embryos, increase transformation efficiency by reducing regeneration time, and that virtually eliminates somaclonal variation. These improvements and modifications are reported here for the first time in detail in a step-by-step procedure for cotton transformation and regeneration that includes commentary for trouble-shooting problems.

***Key words**: Agrobacterium tumefaciens*, Coker, *Gossypium hirsutum*, somatic embryogenesis.

Introduction

Cotton (*Gossypium sp.*) has fulfilled man's basic needs for food and fiber products since its domestication more than 8,000 years ago. Today, cotton remains one of the world's most important economic crop species, and a renewable resource - providing raw materials for textile manufacturing, mulch and cattle feed, as well as a vast array of consumer-based products. The production of genetically modified cotton has been one of plant biotechnology's greatest success stories[26]. Genetically modified cotton, one of the first transgenic crops in commercial production on a large scale, now accounts for the vast majority of cotton acreage in the U.S. and increasingly so in cotton-producing countries around the world. A recently published review on cotton biotechnology provides a critical assessment of gene delivery systems and target tissues for transformation, concluding that the widely used *Agrobacterium*-mediated transformation of tissue explants via somatic embryogenesis is, by far, the most efficient means for generating transgenic upland cotton (*G. hirsutum* L.)[26]. Although the efficiency of *Agrobacterium*-mediated gene transfer is very high[18], the ability to regenerate intact, fertile plants from transformed tissue is another issue. Cotton is recalcitrant to regeneration, and regeneration is strongly genotype-dependent. *Agrobacterium*-mediated transformation of a regenerable line selected from the cultivar Coker 312 and related lines, commonly called "The 'Coker' Method", serves as the industry standard at this time[21-23,25].

Described herein in detail, is a reliable step-by-step protocol for the 'Coker' method of cotton transformation that incorporates numerous improvements and modifications developed in the authors' labs in generating transgenic cotton over the last decade. The procedure addresses the most common problems related to somatic embryogenesis, germination of embryos somaclonal variation, and genotype-dependent regeneration. The general schema for *Agrobacterium*-mediated transformation and regeneration of transgenic cotton plants via somatic embryogenesis is presented in Figure 1. The recovery of transgenic plantlets requires approximately 8 to 10 months and occurs in three discrete stages; 1) Callus Induction, 2) Somatic Embryogenesis, and 3) Germination of Somatic Embryos.

Critical Parameters For *Agrobacterium*-Mediated Cotton Transformation

Genotypes: The genotype-dependent regeneration of fertile transgenic cotton (*G. hirsutum* L.)[2,24] hinges on the regeneration potential of a limited number of related lines[26]. Embryogenic potential is reportedly a genetic trait of low heritability[3,6,9] that varies significantly from cultivar-to-cultivar and from seed lot to seed lot. Moreover, the pedigrees of most, if not all of the regenerable genotypes amenable for transformation using the 'Coker' method, including Coker 312-17, Coker 312-5A, GC510, DP6166, Cascot 2910, Mar 10, and PD lines, share Coker 100W as a common ancestor. A handful of other cultivars exhibit embryogenic potential, but do not exhibit the regeneration efficiency associated with Coker 312, and to lesser extent, its sister lines[2-3,24]. More recently, a novel approach to defining conditions conducive to callus induction and somatic embryogenesis increased the number and diversity of genotypes that can be successfully transformed and regenerated, including Coker and Acala type cotton[9]. Transformation efficiencies in the range of 95-100% are expected using seed from genotypes selected for regeneration potential, whereas commercial seed exhibit 0-30% regeneration potential, and hence, lower transformation efficiencies in the absence of selection.

Explants: Transgenic cotton plants have been successfully regenerated from a variety of tissues including leaves, cotyledons, hypocotyls, shoot tips and embryonic meristems[26]. However, somatic embryogenesis occurs most readily from seedling hypocotyls and to a lesser degree from immature tissues, while mature tissues are generally among the most

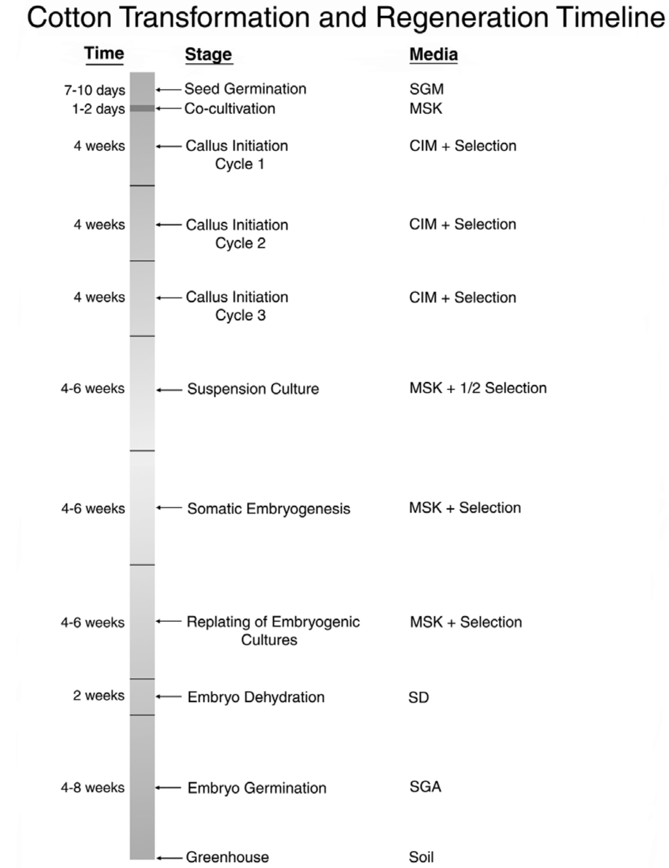

Figure 1. General scheme highlighting the key steps in cotton transformation and regeneration and the medium associated with each stage in the process. The timeline indicates how long is minimally required at each step, resulting in the recovery of plantlets in 8-10 months on average.

recalcitrant[22]. Explants with a high density of gossypol glands are especially problematic and should be avoided. Hypocotyls remain by far, the best tissue for explants and should be prepared from healthy, vigorous seedlings for optimal results.

Agrobacteria strains and co-cultivation: Transformation efficiencies can be profoundly affected by several factors, such as the *Agrobacteria* strain, temperature of co-cultivation, length of time explants are co-cultivated[18], and even the type of construct. The most commonly used *Agrobacteria* strain is LBA4404[12], although supervirulent helper strains EHA105 (Kn^S) and EHA101 (Kn^R)[1,5], and A281[18] are other *Agrobacteria* strains that reportedly work well. In a comparative study[18], transformation events occurred at a rate ~2-fold higher with LBA4404 than EHA105.

Some *Agrobacteria* strains tend to clump in culture more so than others, and it is recommended that overnight cultures be shaken vigorously on a rotary shaker (250-300 rpm) to maintain a homogeneous cell suspension to optimize transformation efficiencies. The dilution of the *Agrobacteria* culture prior to co-cultivation is key to the frequency of transformation and to controlling overgrowth. Cultures in the exponential growth phase are diluted 1:10 with LBA4404, and 1:20 or 1:40 with more virulent EHA strains. Co-cultivation at 21°C is recommended until a "light" halo of bacterial growth appears around the explant in 1 to 3 days. Increasing the cocultivation time increases the frequency of transformation events, but also increases problems associated with overgrowth, especially with EHA strains. Under these circumstances, it is best to establish optimal conditions for co-cultivation by performing a dosage-dependent time course.

Callus induction: Selection of callus for color, size, organization, and texture is central to successful somatic embryogenesis and the efficiency of regenerating transgenic plants (Figures 2 and 3). In fact, a high failure rate in converting callus to embryogenic cultures and somatic embryos is often reported. Although robust somatic embryos can be recovered from hard, compact dark green or white callus, albeit with difficulty, it is for this latter reason that this type of callus is generally considered non-friable and non-embryogenic and is discarded. Friable Coker callus grown under selection should be cream to light green in color. High quality callus usually shows a small amount of red pigmentation resulting from the accumulation of anthocyanins. A friable callus is also one in which the undifferentiated cells are loosely organized, producing a 'soft' callus that is highly granular or 'beady' in appearance and is readily dispersed in liquid medium. The cells themselves should be very small, cytoplasmically dense cells as calli with large, highly vacuolated cells rarely become embryogenic. During the selection and callus proliferation stage, larger calli exhibit the highest survival rates, and calli should *not* be removed from the original explant until callus is at least 'pea-size' (~5mm)[18, 22-23] (Mishra and Wilkins, unpublished data).

The density of explants per plate also influences callus formation. Placement of too few calli results in minimal growth and proliferation, while too many explants stimulates callus proliferation and poses a high risk of overgrowth if explants are placed too close in proximity to one another. In addition, a high density of explants oftentimes results in overproduction of phenolics that, in turn, inhibit callus initiation and proliferation. It is therefore critical to maintain a watchful eye for the overproduction of phenolics during callus selection and proliferation stages. It is important to place explants gently on the surface of the media and to avoid pushing the explant into the medium. At the first sign of phenolic accumulation, observed as a dark brown-black ring around the explants, transfer the explants to fresh medium regardless of the scheduled transfer date.

The hormonal composition of the callus induction medium is also a key factor in the successful regeneration of transgenic cotton plants. Historically, cotton callus induction medium is based on Murashige-Skoog salts containing an auxin and a cytokinin[2, 3, 6, 11, 14, 18, 21-23]. In early work, however, it was determined that 2,4-D is more effective than NAA in producing a high quality callus[22-23]. More recently, a new formulation of callus induction medium for cotton designated as MCIM (MS salts, 100 mg l^{-1} myo-inositol, B5 vitamins (10 mg l^{-1} thiamine-HCl; 1 mg l^{-1} nicotinic acid; 1 mg l^{-1} pyridoxine), 0.75 g l^{-1} $MgCl_2$, 30 g l^{-1} glucose, 10.7 µ M NAA and 0.2 µM 2,4-D, adjusted to pH 5.8 before adding 2.5 g l^{-1} Phytagel) works extremely well in regenerating Coker as well as other genotypes, including Acala cotton[9].

Somatic embryogenesis and germination*:* Most common problems include a low production rate of somatic embryos,

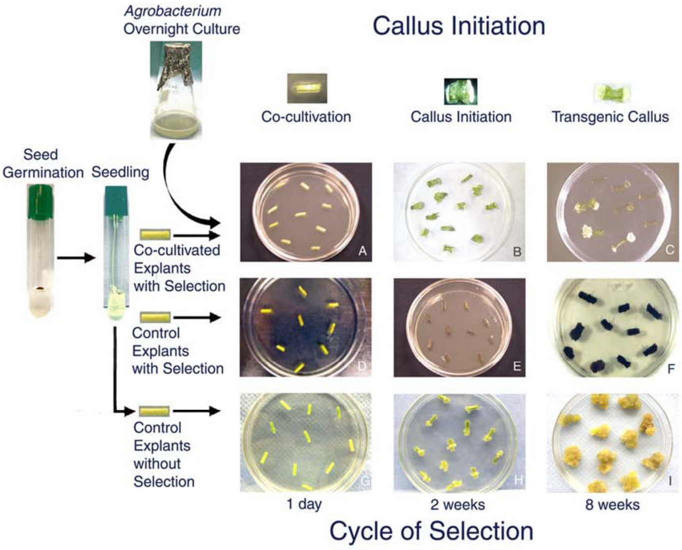

Figure 2. Surface sterilized seeds are germinated 7-10 days in culture tubes. A-C, Hypocotyl explants (~5 mm in length) co-cultivated with a dilute suspension of *Agrobacterium tumefaciens* cultured on MSK medium under selection. Callus formation in ~4 weeks under selection suggests transformation has been successful. After 8 weeks under selection, only a few explants produce surviving transgenic callus (C). D-I, Two experimental controls shown here include explants cultured with (D-F) and without (G-I) selection show that selection is working as expected to produce transgenic calli. D-F, Control explants cultured under selection (including explants co-cultivated with *Agrobacteria* alone (not shown)) become necrotic and die after 2 cycles of selection (F). G-I, Control explants cultured in the absence of selection proliferate friable callus at a much faster rate than co-cultivated explants under selection. Other controls (not shown) include explants co-cultivated with *Agrobacterium tumefaciens* alone and a vector control.

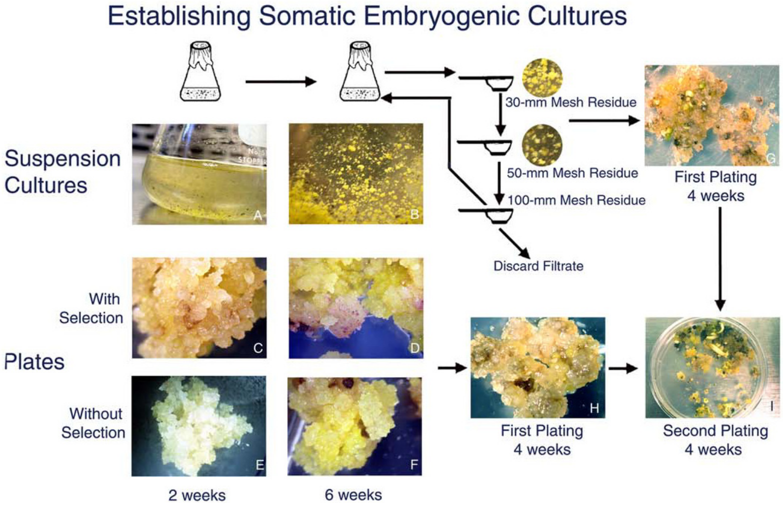

Figure 3. Friable, creamy-colored callus is generally subdivided into 3 parts for transfer to hormone-free MSK media, including (A-B) liquid medium for establishing embryogenic suspension cultures and (C-F) solid medium with or without selection. Growing suspension cultures appear greenish in color (A), and depending on the cell-line, embryogenic suspension cultures (B) are generally established in ~3 to 4 weeks. Selection is added at one-half strength once the cultures are well established. Large cell clumps or organized masses appear in the cultures in 2 to 4 weeks, with well-formed somatic embryos occurring in 4 to 6 weeks (B). Callus transferred to plates used primarily as back-up in case of contamination of suspension cultures also become embryogenic (D, F). A small accumulation of anthocyanin (red pigmentation) in cultures under selection is an indicator of somatic embryogenesis (C-D). Callus grown without selection appear light green in color (E-F). After 6 to 8 weeks, embryogenic suspension cultures are size fractionated by sieving through a series of 30-, 50-, and 100-mm mesh screens to obtain somatic embryos of similar size and developmental stage for synchronizing growth for plating onto semi-solid medium. The 100-mm mesh residue is used to maintain embryogenic suspension cultures. Embryogenic material can be harvested from cultures on plates as well. On the "first" plating, somatic embryos are present in low abundance (G-H). The number, size and quality of somatic embryos increases after the "second" plating (I).

the poor quality of somatic embryos, including a high frequency of abnormal embryos, and a low conversion rate of somatic embryos into plantlets[7]. In the method described here, somatic embryogenesis is promoted in callus cultures by removing exogenous hormones from the medium while maintaining selection. Although somatic embryogenesis occurs readily on plates, establishing embryogenic cell lines in suspension culture is highly recommended as this seems to accelerate morphogenesis, amplifies the number of somatic embryos and produces high quality embryos with a higher survival rate during germination[22-23]. Embryogenic cultures are usually established in ~3 weeks, although some cell lines may take longer to establish. Since somatic embryogenesis is asynchronous and takes place over a period of 3 to 4 months, suspension cultures are maintained by subculturing, with a portion of the callus maintained on plates primarily as a back up. The somatic embryos regenerated from a single suspension culture flask represent the same transgenic event. The reason for amplifying the number of embryos for each event is due to the low efficiency of the process during rooting and germination, which is a limiting factor to cotton transformation efficiencies[26]. However, somatic embryos do develop on plates, and some groups opt to bypass the suspension cultures entirely in favor of plate cultures. Embryogenic cultures contain somatic embryos in varying stages in development that range from globular to torpedo-shaped embryos. High quality embryos are opaque and yellow-green to white in color, whereas amber and transparent ("glassy") embryos should be discarded. Frequent subculturing can reduce vitrification leading to formation of "glassy" embryos. Plating densities of embryogenic cultures is a concern as de-differentiation into callus occurs if the plating density is too high. On the other hand, if the plating density is too low, the somatic embryos fail to thrive and grow. The procedure described in the following section details a strategy for optimizing plating density and recovering somatic embryos.

On average, plantlets are successfully recovered from only 5-6% of somatic embryos[26]. Higher recovery efficiencies are possible, but are highly dependent on the quality of somatic embryos produced. Germination of poor quality somatic embryos is not an efficient process, and is the reason behind the need for producing an excess number of somatic embryos from such cell lines. Inclusion of a dehydration step that mimics seed dormancy promotes a more consistent germination rate among somatic embryos. Smaller embryos may require several transfers on dehydration medium to reach sufficient size to germinate properly. When phenolic compounds are observed, somatic embryos should be transferred immediately to fresh dehydration medium. Once the somatic embryos are transferred to germination medium, and after each transfer to fresh germination medium, the cultures should be placed in the dark for 3 days before returning embryos to light. This routine seems to enhance rooting and reduces the accumulation of excess phenolics.

Light and temperature conditions: A temperature-controlled growth room at 26-28°C with even distribution of heating and cooling is required. For callus cultures, vertically wall-mounted 2- or 4-bulb shop lights equipped with 40-watt cool white (General Electric F40SP41) and 40-watt wide spectrum Gro-lux (Sylvania F40/Gro/AQ/WS/RP) fluorescent bulbs provide indirect lighting on a 16/8 h light/dark cycle. The light fixtures are spaced at 2-foot intervals on the wall at a distance of ~1 foot from the cultures. Continuous direct lighting for plantlets is provided by wall-mounted vertical lights supplemented by overhead fluorescent lights positioned ~24 inches above the culture jars.

Transformation criteria: As a rule of thumb, approximately 300 explants are needed to produce 30 independent transgenic lines, from which the very best lines (10-15) are selected for study. T0 plants (5) from each cell line can be clonally propagated so that a sufficient number of plants (25) can be characterized to minimize variability during the analysis. The formation of callus under selection in 4-6 weeks is considered a successful transformation. The best approach is to perform back-to-back experiments over a period of 1-2 weeks until all explants have been processed. A number of explants (3-5%) should be set aside as transformation controls in each experiment, to include 1) explants (no co-cultivation with *Agrobacterium*), 2) *Agrobacterium* without a construct, and a 3) vector control (*Agrobacterium* + binary vector).

The Coker transformation procedure presented here should produce young transgenic plants in 8 to 10 months (Figure 1) with little to no risk of somaclonal variation. It is critical that cultures are checked daily, and transfers are diligently performed on schedule or earlier if culture conditions demands it. Minimizing time spent in culture is the key to eliminating the risk of somaclonal variation. As the length of time in culture increases, so does somaclonal variation, a problem often associated with early methods requiring 12-15 months to regenerate transgenic cotton plants[2,13,15].

Seed Processing

The seed source is a major factor in controlling seed-borne contaminants, which pose a major problem in terms of transformation efficiency. Seed harvested from greenhouse-grown plants are preferred over commercial seed in terms of seed quality, germination rates, and seedling vigor. In general, overall quality of seed harvested from winter nurseries is compromised, and results in lower transformation efficiencies. Even acid delinted field-grown seed are notoriously difficult to sterilize by current methods, and contamination by soil-borne fungal pathogens oftentimes gains the upper hand. These problems are minimized in greenhouse-produced seed and are therefore preferred over field-grown seed, although new, simpler methods of sterilization can overcome contamination problems.

Test each new seed lot for germination rate and rate of fungal/bacterial contamination before and after acid delinting seed, as well as after surface sterilization of seed. These tests aid in determining the best seed sterilization method for each seed lot, and in calculating the number of seeds needed to provide a sufficient number of healthy seedlings and hypocotyl explants.

The general method described below is usually sufficient for sterilizing seed harvested from greenhouse-grown plants. However, this method is not sufficient to eliminate fungal growth commonly associated with field-grown or commercial seeds.

1. Add 30-50 acid delinted seeds to a sterile 150-ml flask containing 100 ml of a 20% bleach (v/v) solution supplemented with a drop of Tween 20 as a surfactant.

2. Add a magnetic stirring bar to the flask, loosely cover the flask with foil, and agitate the seeds on a magnetic stirrer for ~20 minutes in a fume hood.

3. In a laminar flow hood, rinse the seeds 5 times with sterilized ddH$_2$O. Prepare 5 500-ml flasks containing 300 ml of sterile ddH$_2$O and sterile 50-mm mesh stainless steel sieves in advance.

    3.1. Collect seed in a sterile sieve. Using a spatula, transfer seed in the sieve to the first 500-ml flask. Briefly rinse the seeds by swirling the flask for ~1 minute.

    3.2. Collect seeds in a sterile sieve and repeat process until seeds have been washed a total of 5 times. Following the final wash, dry the seeds for 30 minutes on sterile filter paper. The sterile seeds may be stored in a sterile petri dish sealed with parafilm for 2-3 days, or germinate immediately.

*Agrobacterium*mediated Transformation of Cotton Hypocotyl Explants – Step-by-Step Protocol

The following procedure describes a general transformation protocol (Figure 1) for a binary vector containing *nptII (neomycin phosphotransferase II),* the gene conferring kanamycin resistance as the selectable marker, and the *Agrobacterium tumefaciens* strain LBA4404.

1. Preparation of hypocotyl explants: To recover a sufficient number of transgenic plants for testing and evaluation in a research setting, it is recommended to do transformation experiments in stages to obtain ~300 explants per construct. For example, from 50 seedlings, use 30 seedlings (~300 explants) for the test construct, 3-5 seedlings for the vector control, and 3-5 seedlings for each of the untransformed controls (with or without LBA4404) (see Figure 2). Repeat the co-cultivation each week for the next 2 weeks. It takes at least 4-6 weeks to determine if the transformation was successful.

1.1.Prepare 25 X 150-mm culture tubes containing 15 ml of SGM medium to obtain 50 seedlings free of contamination.

1.2.Place 1 surface-sterilized seed per culture tube. Germinate seeds by incubating culture tubes at 28-30^{0}C under indirect lighting for ~7-10 days, or until the hypocotyls are 7-8 cm in length (Figure 2).

1.3.Using a pair of long, sterile forceps, remove seedling from culture tube. Cut off the cotyledons, shoot tip and roots with a scalpel.

1.4. In a sterile petri dish, use a scalpel to dissect the hypocotyl into sections ~5- mm in length. Each seedling will yield 8-10 explants Transfer explants to a sterile petri dish (100-150 per 25 X 100-mm plate) containing a few drops of sterile water to prevent drying. Store the untransformed and vector control explants in separate petri dishes.

Note: *Vigorous seedlings with "fat" hypocotyls that "snap" when slicing are the best explants for Agrobacterium-mediated transformation. Discard any 1) spindly seedlings or hypocotyls, 2) plants with thin, yellow roots which often harbor endocytic bacteria, and 3) plants in which the tap root is dead or fails to grow into the media. The health and vigor of the seedling is key to successful contamination-free transformation.*

2. Co-cultivation of hypocotyl explants: During germination of seedlings (Section 1), streak fresh plates of the *Agrobacterium tumefaciens* strain LBA4404, the construct in LBA4404, and LBA4404 containing the binary vector only (vector control) from -80°C freezer stocks on YEP medium supplemented with the appropriate antibiotics. Incubate inverted plates at 28°C for 1-2 days.

Note: If the colonies vary in size after long-term storage in the freezer, re-streak fresh plates and incubate for 1-2 days at 28°C to obtain colonies more uniform in size. Plating Agrobacterium is preferred over direct inoculation of cultures for co-cultivation from freezer stocks.

2.1.Set-up overnight cultures by inoculating 25 ml YEP (or LB) + antibiotics in a sterile 50-ml flask with 3-4 colonies.

2.2. Incubate culture overnight at 26-28°C, shaking vigorously at ~250 rpm to minimize clumping of cells, which is especially problematic with some strains of *Agrobacteria* (Figure 2).

2.3. Measure the OD_{600} of the overnight culture (~1 ml) using YEP or LB + antibiotics as the background reference (blank). Dilute the culture to an $OD_{600} = 0.3$. Incubate the diluted culture at 26-28°C on a shaker at ~250 rpm for approximately 3-4 hours to an $OD_{600} = 0.6$.

2.4. Prepare a 1:10 dilution of the culture with MSK.

Note: A 1:10 dilution may produce overgrowth of Agrobacterium following co-cultivation with some strains such as EHA101. In these cases, use dilutions of 1:20 or 1:40, or perform a test to determine what dilution factor is most suitable. Optional: *To induce* vir *genes, add acetosyringone suspended in DMSO to Agrobacterium cultures (Section 2.2) to a final concentration of 100 µM prior to co-cultivation*[18].

2.5. Remove water from petri dish containing 5-mm hypocotyl sections and add diluted *Agrobacteria* culture to cover the explants for 30 minutes. Co-cultivate 2 plates containing 100 150 explants with LBA4404 + construct, 1 plate (25-50 explants) with LBA4404 as the "Agro" control, and 1 plate (25-50) explants with LBA4404 + vector as the vector control. For the untransformed (no *Agrobacteria*) control, cover explants with MSK medium for 30 min.

2.6. Aspirate *Agrobacteria* and let explants air dry, or transfer explants to a petri dish containing dry sterile filter paper to blot excess *Agrobacteria*. Blot untransformed controls on filter paper as well.

2.7. Transfer and uniformly distribute ~8-10 explants per 20 X 100-mm plate (Figure 2) containing preferred callus induction medium (e.g., CIM). Incubate unsealed plates in the dark at 21-22°C for 2 to 3 days.

Note: Explants should be surrounded by a 'halo' of Agrobacteria growth. A dense, viscous 'halo' indicates overgrowth of Agrobacterium, and it is more expedient to discard 'overgrowth' explants, or abandon the experiment if the problem is widespread. If overgrowth is an issue, use a more dilute culture of Agrobacteria for co-cultivation.

3. Callus initiation: This phase will take approximately 3 to 4 months to complete, and requires at least 3 transfers at monthly intervals to produce sufficient kanamycin-resistant callus for establishing cell suspension cultures (Figure 1). The CIM callus induction medium is the standard for Coker regeneration. Alternatively, MCIM works extremely well with Coker as well as other genotypes[9].

3.1.Blot co-cultivated explants on dry sterile filter paper and transfer to CIM (or MCIM) callus induction plates supplemented with 50 ì g ml^{-1} kanamycin + 500 ì g ml^{-1} carbenicillin (8-10 explants per 25 X 100-mm plate). Control explants should be divided between plates with and without selection (Figure 2).

Note: If there is a problem of overgrowth, use carbenicillin (400 ì g ml^{-1}) plus claforan (or cefotaxime) (200 ì g ml^{-1}). Caution - some Agrobacterium strains contain an amp gene and are resistant to carbenicillin.

3.2. Seal plates with parafilm and incubate at 28°C under indirect lighting to induce callus formation (Callus Initiation – Cycle 1). Check plates daily. After 3 to 4 weeks, callus will appear as small, white growth forming at the cut-end of the explants (Figure 2).

3.3.Transfer explants to fresh CIM selection (50 mg ml^{-1} kanamycin + 500 mg ml^{-1} carbenicillin). Transfer control explants to separate plates with or without selection. Seal plates with parafilm and incubate at 28°C for an additional ~4 weeks (Callus Initiation – Cycle 2). Kanamycin-resistant callus will range from a pale, dull cream color to yellowish-green to snowy white, depending on the size and quality of the callus (Figure 2).

Note: If callus at each end of the explant is large enough (>2 mm), cut the explant in half and transfer each half of the explant to fresh plates. Each end of the explant should represent~ an independent transformation event (cell line) and should be labeled accordingly.

3.4. Transfer explants/callus onto fresh CIM selection plates and incubate at 28°C for ~4 weeks (Callus Initiation – Cycle 3). Discard dead explants. For control explants, only the vector controls should proliferate callus under selection, while all control explants should produce callus on CIM plates without selection (Figure 2).

Note: If kanamycin-resistant callus are too small (<2 mm), subculture for 2-4 weeks on callus induction medium without selection to proliferate callus prior to establishing embryogenic suspension cultures (Section 4). Three cycles of selection has proven sufficient to essentially eliminate non-transgenic cells ('escapes') and problems with chimeras. Adding this step increases the success rate of transformation efficiency and conversion of callus to embryogenic cultures.

4.Somatic embryogenesis:

4.1. Transfer 1/3 of the callus to MSK-kanamycin, and 1/3 of the callus to MSK plates without selection (Figure 3). Seal plates with parafilm and incubate at 28°C under indirect light. Maintain only 1 cell line per plate at this stage. Subculture at 3- to 4-week intervals. These plates serve primarily as back up in case suspension cultures become contaminated.

Note: Although the protocol described here opts for establishing embryogenic suspension cultures (Section 4.2), these cultures do undergo somatic embryogenesis and many groups bypass the suspension cultures entirely in favor of plating. Inclusion of kinetin (0.1 mg L^{-1} cytokinin) and auxin (2mg L^{-1} NAA) is oftentimes included in this case to promote somatic embryogenesis[2, 18].

4.2.To establish suspension cultures for each cell line, transfer 1/3 of friable callus to a sterile foil-covered 250-ml flask containing MSK (40 mg fresh weight of callus per ml of MSK media).

4.3. Incubate flasks at 28°C on a rotary shaker at 120 rpm under indirect lighting for 10-15 days, or until the culture is established and actively proliferating before adding ½ strength kanamycin (25ì g ml^{-1} final concentration). After ~3-4 weeks, the culture should be embryogenic and ready for plating onto semi-solid MSK medium, although some cell lines may take ≥4-6 weeks.

Note: Cultures are normally dark in color (Figure 3). *Somatic embryogenesis is asynchronous as the rate at which embryos are produced will vary between cell lines and not all cultures will be ready for plating within this time frame. If not, continue growing until cultures become embryogenic. Continually monitor callus on MSK plates under a dissecting microscope to look for diagnostic multicellular clusters that resemble a wheel with spokes or a grape cluster. Embryogenic cultures contain very small globular embryos observed as small beads with a smooth epidermal surface. Embryogenic cultures should be rich in small globular somatic embryos (Figure 3 and 4). Cultures are now ready for plating onto semi-solid media to promote further development of somatic embryos. Close examination of callus cultures on solid MSK plates should also show onset of embryogenesis, and these cultures can now be put into suspension cultures for proliferation or transferred directly to semi-solid plates along with the suspension cultures.*

5. Sieving embryogenic suspension cultures: Size fractionation of embryogenic suspension cultures cells as shown in Figure 3 has several distinct advantages, including 1) exclusion of non-embryogenic cell clumps, 2) synchronizing development of somatic embryos, 3) recovering cells for maintaining embryogenic suspension cultures, and 4) amplification of the number of high quality somatic embryos for germination.

5.1. Decant suspension culture through a sterile stainless steel 30 mm mesh sieve. Capture filtrate (flow-through) in a sterile petri dish and set aside for the next sieving step. Discard cell debris, and hard white or dark green aggregates with a pair of forceps, as these clumps tend to produce fast-growing, non embryogenic callus.

5.2.Transfer the cells retained by the 30-mm mesh sieve to a sterile 50-ml polypropylene tube with a sterile spatula.

5.3.Add 15-20 ml of MSK, gently swirl the tube to wash the cells, and set aside in a rack to let the cells settle for 2-3 minutes. Decant media and repeat wash step. Save 30-mm mesh residue for plating (Section 6).

5.4.Sieve the 30-mm filtrate through a 50-mm mesh sieve and wash the cells as described (Section 5.1-5.3). Save the 50-mm residue retained by the mesh sieve for plating (Section 6).

5.5.Sieve the 50-mm filtrate through a sterile 100-mm mesh sieve. Discard the 100-mm mesh filtrate.

5.6. To maintain suspension cultures, wash the 100-mm mesh residue as described (Section 5.1-5.3) and transfer the 100 mm cells retained by the sieve to a 50-ml polypropylene tube and add MSK to a final volume of 50-ml. Transfer to a 250-ml flask and incubate at 120 rpm under indirect lighting as described (Section 4.3).

6. Plating embryogenic suspension cultures on semi-solid medium:

6.1. Suspend washed 30- and 50-mm embryogenic cells from steps 5.2 - 5.4 in MSK at a ratio of 9-ml media per ml of settled cells using a wide-bore pipet to disperse the cells.

6.2. Swirl the tube, transfer 2-ml aliquots using a wide-bore pipet to MSK plates containing 25 ì g ml^{-1} kanamycin. Gently rotate the plates to uniformly distribute the cells (Figure 3).

6.3. Repeat the process until all cells are plated, including two plates without antibiotics to monitor the efficiency of selection. Leave covered plates (~10-15) in a laminar flow hood overnight, or until the excess liquid media from plating has been absorbed or evaporated.

6.4. Seal covered plates with parafilm and incubate under indirect lighting at 28°C for 4-5 weeks. Monitor plates daily to observe the formation and growth of somatic embryos. Occasionally "stimulate" cultures by gently tapping the plates to relocate embryonic tissue to new sites on the plate. This is especially important if development appears slow and large clumps begin to look somewhat "glassy".

6.5. Select the best material from each cell line for re-plating (subculturing or "second" plating). Wash cells as described (Section 5.3-5.4). Suspend embryogenic cells in MSK and re plate as described in preceding steps (Sections 6.1-6.4).

Note: To select material for second plating, harvest embryogenic "nests" and surrounding cells with sterile forceps. Gently spread the remaining callus with the forceps, and continue to harvest. Embryogenic "nests" are soft, loosely organized cell clusters that appear "shiny", granular in texture – resembling clusters of balloons or grapes, and vary from creamy to light green in color. This step is critical for the successful and efficient recovery of transgenic plants.

6.6. Leave covered plates overnight in a laminar flow hood as in Section 6.3. Seal covered plates with parafilm and incubate under indirect lighting at 28°C for ~3-4 weeks. Continue rounds of subculturing and plating to maintain embryogenic cultures.

7. Germination of somatic embryos: After the second plating of embryogenic cultures (Section .6.5-6.6), the cultures will be highly enriched in somatic embryos at varying stages of development (Figure 4). Select healthy, yellow-green opaque heart- and torpedo-shaped embryos showing a smooth epidermal surface for germination. Many cell lines will form globular embryos that only increase in size and do not develop further. Such embryogenic cultures are characterized by cell clusters with rough, dull surfaces often resembling the surface of cauliflower. This indicates callus formation, and embryogenic cultures that will usually continue to form only secondary embryos without further manipulations. Other embryogenic tissue or embryos that are dull, white, very dark, hard, "crunchy", translucent, "glassy", amber-colored or any combination thereof are not desirable and should be discarded.

The idea behind a "dehydration" step is to reduce the moisture content to mimic seed dehydration as a means of increasing the success rate of germinating somatic embryos. During this stage, the embryos continue to increase in size, and the region of root formation becomes brown in color (Figure 4). Many somatic embryos may begin to develop roots. If somatic embryos are >0.5 cm in size, torpedo-shaped, and/or are becoming bleached, omit the dehydration step and proceed directly to germination (Section 7.4).

7.1. Transfer yellow-green heart-shaped embryos (≥5 mm) (Figure 4) to SD plates (~15 embryos per plate). Incubate covered plates in the dark at 28°C for ~10-15 days.
Do *not* seal plates in parafilm, and check plates daily to ensure that media does not completely desiccate. Somatic embryos will continue to grow on dehydration medium.

7.2. Transfer dehydrated somatic embryos to SGA plates for germination (~15 embryos per petri plate). Do *not* seal plates with parafilm. See Figure 4.

7.3. Incubate plates in the dark at 28°C for 3 days before placing plates under direct light. Allow media to dry out slowly over a 2-3 week period, but monitor daily to avoid complete desiccation. Root formation should occur once embryos produce the first true leaves.

7.4. Remove old, dark roots with a scalpel to enhance shoot development. Transfer germinating somatic embryos to fresh SGA medium every 6-8 weeks or sooner if media becomes too desiccated, or if the first true leaves develop. Incubate germinating embryos for 3 days in the dark after each manipulation before returning the embryos to direct light.

7.5. Transfer small plantlets to pint jars containing 45 ml of SGA medium (1 plantlet per jar) and incubate under direct lighting at 28°C for ~4-6 weeks until plantlets are well established with sufficient root system and 4-6 leaves (Figure 4).
Optional: Clonally propagate the plant in pint jars of SGA medium. Maintain the parent clone in culture until the clones

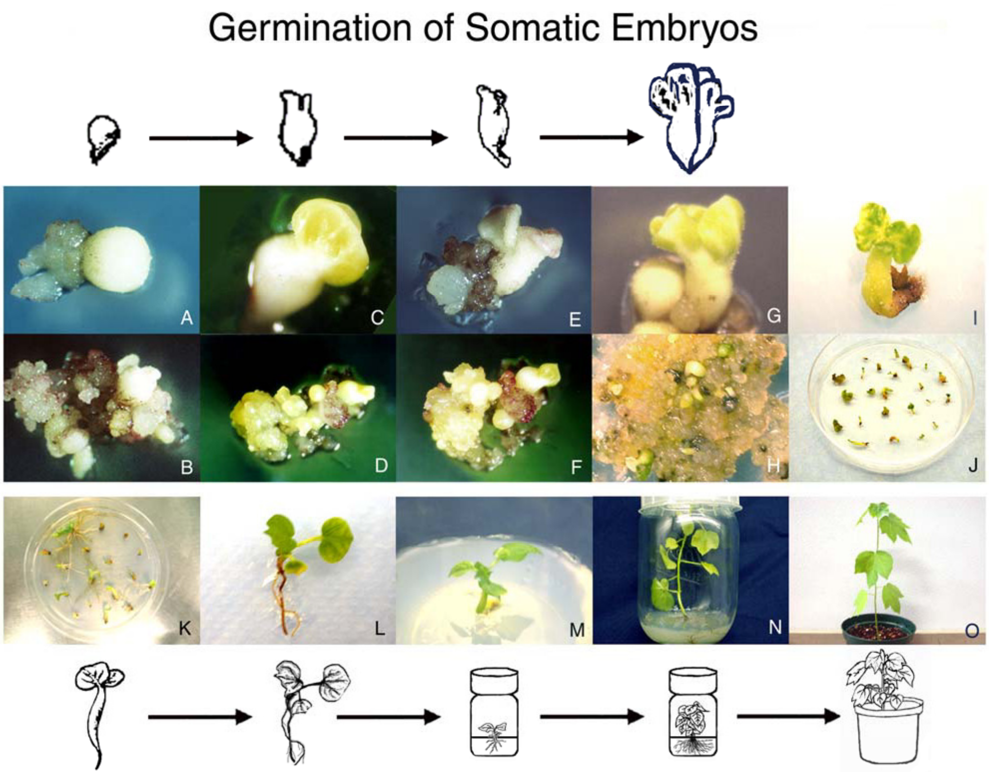

Figure 4. Embryogenic cultures harbor embryogenic clusters and nests of somatic embryos. A-B, Globular-shaped somatic embryos are the first to appear in embryogenic cultures (Figure 2). C-H, Heart-shaped somatic embryos (C-D) begin to initiate formation of a root zone (E-F). I-J, Heart-shaped somatic embryos (>5 mm) are passed through a dehydration step that mimics seed dormancy. K-L, Torpedo-shaped and desiccated heart-shaped embryos are transferred to germination medium where roots will begin to develop over a period of 2 to 4 weeks. M-N, Once roots are established and the first true leaves have formed, the "seedlings" are transferred to pint jars. O, Once well established, plantlets are planted in soil and transferred to the greenhouse.

mature in the greenhouse to ensure recovery of each independent transformation event.

7.6. Once the plantlet has 5-6 vigorous white roots ~2 in. in length, remove the plantlet from the jar and gently rinse roots in ddH_2O to remove agar.

7.7. Transfer plantlets to soil (Figure 4) and cover with a polyethylene bag. "Harden off" plants by gradual exposure to the environment over a period of 12-15 days by making incisions in the protective plastic bag until the plant is fully exposed. Transgenic plants bloom in 4-6 months.

Soltions and Reagents

Modified Stewart's germination medium (SGM), pH 6.8

10 ml	100X Stewart's Micronutrient Solution (Stewart and Hsu [16])
20 ml	50X Stewart's Macronutrient Solution (Stewart and Hsu[16])
10 ml	100X Stewart's Vitamin Solution (Stewart and Hsu[16])
3.68 mM	$MgCl_2 \cdot 6H_2O$ (FW 203.31)
0.5% (w/v)	Sucrose
0.2% (w/v)	Phytagel (Sigma P-8169)
0.5% (w/v)	DIFCO Bactoagar

SGA, pH 6.8

SGM medium supplemented with 5.7 mM IAA (1.0 mg ml^{-1} in 95% ethanol stored at -20^0 for 〈 2 months).

50X B5 vitamin stock

0.50 g thiamine-HCl (FW 337.3)

0.05 g nicotinic acid (FW 123.1)

0.05 g pyridoxine HCl (FW 205.6)

Callus induction medium (CIM), pH 5.8

4.3 g L^{-1}	Murashige-Skoog (MS) salts (GibcoBRL Cat No. 11117-074)
100 mg L^{-1}	Myo-inositol
0.05X	B5 vitamins (50X stock)
3.0% (w/v)	Glucose
3.68 mM	$MgCl_2 \cdot 6H_2O$ (FW 203.31)
0.452 mM	2,4-D (1.0 mg ml^{-1} in 95% ethanol stored at -20°C for < 2 months)
0.464 mM	Kinetin (1.0 mg ml^{-1} in 95% ethanol stored at -20°C for < 2 months)
0.2% (w/v)	Phytagel

MSK, pH 5.8

4.3 g L^{-1}	Murashige-Skoog (MS) salts
100 mg L^{-1}	Myo-inositol
0.05X	B5 vitamins (50X stock)
3.0% (w/v)	Glucose
3.68 mM	$MgCl_2{\cdot}6H_2O$ (FW 203.31)
1.87 mM	KNO_3
0.2% (w/v)	Phytagel

Stewart's dehydration medium (SD), pH 6.8

10 ml	100X Stewart's Micronutrient Solution (Stewart and Hsu[16])
20 ml	50X Stewart's Macronutrient Solution (Stewart and Hsu[16])
10 ml	100X Stewart's Vitamin Solution (Stewart and Hsu[16])
3.68 mM	$MgCl_2{\cdot}6H_2O$ (FW 203.31)
1.0 % (w/v)	Sucrose
0.2% (w/v)	Phytagel
1.5% (w/v)	DIFCO Bactoagar

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Figure