Agrobacterium-mediated Sorghum Transformation

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Plant Material

Immature embryos from Sorghum bicolor cv. P898012 are used for transformation. Plants are grown under standard greenhouse condition until flowering. Sorghum plants are self-pollinating and are bagged with a pollination bag as soon as the flower inflorescence is fully emerged.

Embryos should be between 1.4-1.6mm when used for transformation (~13-18 days past anthesis of the terminal flower). Monitor and inspect embryos prior to harvest to ensure the correct size. The size of the embryo is the most important factor in staging of the transformation experiment. If the embryo is too small it will not survive transfection, and if it is too large it cannot be removed easily without breaking. Broken or damaged embryos will not produce embryogenic callus.

Genotypes: Sorghum bicolor cv. P898012
others are possible at low frequencies

Laboratory Supplies and Stock Solutions (See Appendix 1)

Initiation of *Agrobacterium tumefaciens* culture:

1. Streak out *Agrobacterium* culture from -80°C glycerol stock onto YEP medium plate containing appropriate antibiotics and place the plate at 25°C for 4 days.

2. Add 25 ml of sterile infection medium into 50 ml Falcon tube. Add acetosyringone (100 mM/mL stock solution) to the each of the tubes containing infection media for a final concentration of 100 µM/mL acetosyringone.

3. Take about 2.5 full loops from YEP plate and suspend in the tube. (about 1 full loopful/10ml of infection medium.

4. Shake the infection medium + agro at 25°C at 175 rpm for 1 hour to re-suspend fully.

5. Transfer 2 ml of such suspension to spectrophotometer curette and measure O_{D600} =~ 0.30-0.50 (1 X 1^{09} cfu/ml).

6. Continue shaking the re-suspended culture in a shaker at 175 rpm at room temperature until infection. (~2-3 hours)

7. While the *Agrobacterium* infection cultures are being re-suspended, aliquot 2 ml of infection medium +acetosyringone into sterile 2 ml micro-centrifuge tubes. *Note: These tubes will be used to collect the immature embryos. Make sure that all centrifuge tube racks are thoroughly surface sterilized using 70% ethanol prior to use.*

Embryo isolation and infection

1. Sample several caryopses from the inflorescence prior to harvest to determine the size of the immature embryos about 13 days past anthesis. The embryos should be 1.4-1.8mm. Embryo size will vary depending on their location on the inflorescence.

2. Immature caryopses are removed by hand and placed into a 50 ml Falcon tube.

3. Surface sterilize the isolated sorghum caryopses with 50% bleach (some protocols add a few drops of Tween as a wetting agent) followed by shaking at 200 rpm at room temperature for 30 min.

4. Rinse the caryopses with sterile ddH_2O 3 times or until there is no odor of bleach. After the final rinse remove all of the water.

5. Transfer the sterilized caryopses from Falcon tube (a sterile spatula or forceps may be used) and place into sterile, empty Petri dishs containing sterile Whatman filter paper. Moisten filter paper with a few drops of sterile distilled water. Depending on the size of the experiment, aliquot surface sterilized caryopses into several Petri dishes with the moistened Whatman filters . Aliquots will prevent desiccation of any one sample during embryo isolation.

6. Following transfer of the surface sterilized caryopses into several Petri dishes place covers onto the Petri dishes to prevent desiccation during embryo isolation.

7. Working in the sterile Petri dish and under a dissecting microscope, use 2 fine jewelers forceps to slit the caryposis and carefully remove the immature embryo. The immature embryo is opposite the divot in the caryopsis. *If the embryos are too large, they will be sticky and hard to remove. The embryo size should be 1.4-1.6mm.*

8. Place embryos into the sterile 2.0ml micro-centrifuge tube containing infection) medium+ acetrosyringone (100uM) that was previously prepared . Continue to collect embryos into the micro-centrifuge tube for no longer than 20 minutes to prevent hypoxia of the embryos. *Note: It is important to not let the embryos stay in the liquid media too long. Set a timer to help.*

9. As mentioned previously this procedure is best accomplished with two people, to minimize the time the embryos are submerged in the infection medium. After 20 minutes, transfer the capped tube containing the isolated embryos in the infection media to the partner. The person working on the embryo isolation can start on the next tube.

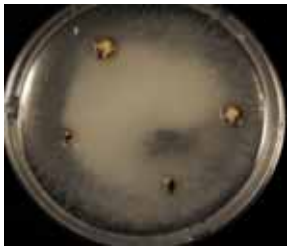
10. The partner should then remove the infection medium from the micro-centrifuge tube and replace with the infection medium containing the *Agrobacterium* suspension. Let stand for 5 minutes for inoculation.

11. The partner should then transfer the embryos from the tube onto a Petri plate containing the Co-cultivation medium. Remove the excess suspension and orient the embryos scutellum side up. A sterile spatula (or forceps) can be used for this procedure, taking care not to damage the embryos. Allow the plates to sit ajar with no lid for up to 30 min in the laminar flow hood to dry excess remaining infection medium. This will help prevent the *Agrobacteirum* from overgrowth during the co-cultivation period (there should not be any liquid surrounding the embryo prior to wrapping the plates). Wrap the plate with parafilm and place them in a tissue culture incubator at 25°C in dark for 3 days. This time for incubation may vary depending on the concentration of Agro in the inoculation and the agro strain.

Resting

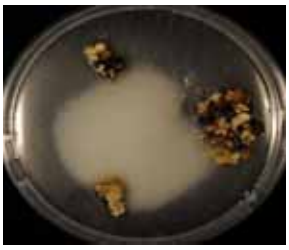
1. After the 3 day co-cultivation period, transfer embryos to Resting medium and incubate for 7 days at 28°C in dark . The embryos should be slightly yellow, swollen and firm. If the embryos are flaccid and white, do not transfer. Count the number of embryos. This will be the initial number of embryos infected when calculating transformation efficiency.

Selection



1. Following the Resting phase, embryos are transferred to Selection I medium. Seal the plates with parafilm and incubate them at 28°C in dark . Due to high phenolic accumulation in the medium, the embryos should be sub-cultured every 5-7 days. Putative transformants should be visible after 6-8 weeks. *Note: It is important to not allow the phenolic zone around the embryo to grow too large. Frequent subcultures are very, very important.*

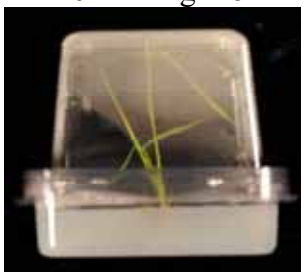
2. Once putative transformants/herbicide resistant calli can be identified, they are transferred to Selection II medium and incubated at 28°C in dark for 2-3 weeks to develop somatic embryos; transferring weekly. *Note: Frequent subcultures are necessary to preserve callus health.*



3. After somatic embryos are visible, transfer callus to Regeneration I medium for 2-3 weeks to develop shoots. Incubate at 28°C under 16 hour light/8 hour dark. Shoots should be well formed and 3-5 cm tall before transferring to Regeneration II media.

Regeneration

1. Remove the small shoots of 3-5 cm tall and cut apart with a scalpel to individualize plants. If plants are not separated here, it will be very hard to transplant into soil. Transfer shoots to Regeneration II medium in Plantcons. Incubate at 28°C under 16 hour light/8 hour dark (low intensity light).



2. When plants reach 8-10cm tall with large, healthy roots systems they can be acclimated to soil.

Acclimation to soil:

3. Once plants are well formed with shoots and roots, they are acclimated to soil. Up to 10 clones from each event are planted to ensure that seed is obtained.

4. Individual plants are planted into well moistened Metromix 510 in 5 gallon pots. Excess media is carefully washed away by dipping roots into room temperature water to minimize fungal growth. Plants are immediately cover with an empty plantcon top to ensure the high humidity that the plants are accustomed to is maintained. *Plants have a very thin cuticle after tissue culture and care must be taken to prevent desiccation.*

5. Plants are place in trays, watered thoroughly and placed in the growth chamber under shade cloth at 27°C under sodium halide lighting. Sorghum is slowly allowed to acclimate to the higher light. After 4-5 days, plantcon tops are propped up slightly to allow plants to begin to develop a thicker cuticle. Plantcon tops can typically be removed after 10 days.

6. Plants are grown to maturity and selfed. T1 seed is collected at maturity.

Sorghum Medium Recipes (1L)

Component	Infection	Co-cultivation	Resting	Selection I	Selection II	Regeneration I	Regeneration II
MS salts	2.15g	2.15g	4.3g	4.3g	4.3g	4.3g	2.15g
MES		0.5g	0.5g	0.5g	0.5g	0.5g	
L-proline		0.7g				0.7g	
Sucrose	68.5g	20g	30g	30g	30g	60g	30g
Glucose	36g	10g					
2,4-D 1mg/ml	1.5ml	2ml	2ml	1.5ml	1.5ml		
Agar		8g				8g	
Phytagel			2.5g	2.5g	2.5g		2.5g
pH	5.2	5.8	5.8	5.8	5.8	5.6	5.6
Autoclave	filtered	20min	20min	20min	20min	20min	20min
B5 vitamin stock, 100X	10ml	10ml	10ml	10ml	10ml		
Acetosyringone, 100mM	1ml	1ml					
Ascorbic acid		10mg	10mg				
Casamino acids	1g						
Asparagine			0.15g				
Coconut water			100ml				
Carbenicillin			0.2g	0.2g	0.2g	0.2g	0.2g
PPT, 20mg/ml				0.25ml	0.5ml	0.5ml	
Kinetin, 1mg/ml					0.5ml		
Zeatin, 1mg/ml						0.5ml	
IAA, 1mg/ml						1ml	
ABA, 0.025mg/ml						1ml	
TDZ, 0.5mg/ml						0.2ml	
IBA, 1mg/ml							0.25ml
NAA, 1mg/ml							0.25ml
MS vitamin, 1000X						1ml	1ml
PVPP (1% final)		10g	10g	10g	10g	10g	5g

B5 Vitamin stock recipe	100X	Final
Myo-inositol	10g	0.1g/L
Nicotinic acid	0.05g	0.5mg/L
Pyridoxine-HCl	0.05g	0.5mg/L
Thiamine-HCl	0.1g	1.0mg/L

Appendix 1

Laboratory Supplies

a. 5 L pitcher or beaker (Phytotechnologies Laboratories B265 or similar)
b. Heavy weight, food service aluminum foil (Phytotechnologies Laboratories F041 or similar)
c. Stainless steel spatula/spoon (Phytotechnologies Laboratories S978 or similar)
d. Stainless steel micro spatula(s), 165 mm, with tapered end
e. Stainless steel scalpel handles (Phytotechnologies Laboratories S963 or similar)
f. No. 11 carbon steel scalpels (Phytotechnologies Laboratories S971 or similar)
g. Stainless steel, serrated tip, utility forceps (Phytotechnologies Laboratories F950 or similar)
h. Clear, sterile, polystyrene petri dishes, 60 × 15 mm (Phytotechnologies Laboratories D940 or similar)
i. Commercial Bleach (e.g., Chlorox)

Stock Solutions

a. Acetosyringone (100mM)- dissolve 19.6 mg in ~ 500 µL of DMSO (dimethyl sulfoxide); once dissolved, bring the volume 1 mL and filter sterilize into a sterile 1.5 mL microcentrifuge tube. This can be scaled up for larger batches, but is typically sufficient for 10 + transformation experiments. can be stored frozen at - 20°C for up to 30 d.

b. 100 mM Silver nitrate ($AgNO_3$) – dissolve 850 mg in ~40 mL of ddH_20. Once dissolved, bring the volume up to 50 mL and filter sterilize into a sterile 50 mL tube. Wrap the tube with aluminum foil and store at 4 °C for up to 1 year.

If used:

c. L-Cysteine (L-Cys) – dissolve 400 mg in ~ 3 mL of ddH_20 by affixing the tube to a vortexer/mixer. Shake vigorously until the all the L-Cys is dissolved. Bring the volume up to 4 mL. Filter sterilize the resulting solution (100 mg/mL), and add the appropriate volume to reach a concentration of 400 mg/L co-cultivation medium. Make this fresh for every batch of co-cultivation medium; a transformation experiment using 6 well pollinated ears in our experience requires 12 – 15 co-cultivation plates or 300 – 400 mL total medium, so preparing 200 mg in 2 mL as described above is often the best approach.

d. DL-Dithiothreitol ("DTT" – 1,4- Dithiothreitol) – dissolve 150 mg in ~500 mL ddH_20. Once dissolved, bring the volume 1 mL and filter sterilize into a sterile 1.5 mL microcentrifuge tube. Add the appropriate volume to reach a concentration of 150 mg/L co-cultivation medium. As in the case of L-Cysteine above, make this stock fresh for every batch of co-cultivation medium.