Brachypodium transformation

John Vogel, modified 2/13/2008
Reference for this method:
Vogel, J. and Hill, T. (2008) High-efficiency *Agrobacterium*-mediated transformation of *Brachypodium distachyon* inbred line Bd21-3, Plant Cell Reports, in press. DOI 10.1007/s00299-007-0472-y

Note: Modifications to the published method are briefly listed below.

- Addition of $CuSO_4$ to the CIM
- Eliminate the addition of water to the filter paper in petri dishes used for the 3 day cocultivation/dessication step
- Transfer callus pieces to selective media after the 3 day cocultivation/dessication step.
- Begin moving callus to regeneration media at after 3 weeks selection rather than after 4.

Callus initiation from excised embryos

Materials needed:
Bleach (5.25% NaOCl)
10% Triton X-100 stock solution
22.5 mM (5 mg/ml) 2,4-D stock solution (2000 X stock)
dissolve 50 mg 2,4-D in 10 ml 95% ethanol
0.6 mg/ml $CuSO_4$ (1000X stock)

Callus initiation media (CIM) per L:
4.43 g LS salts
30 g sucrose
1 ml 0.6 mg/ml $CuSO_4$
pH 5.8 with 0.1 N KOH
2g phytagel for plates (be sure bottles are dry before adding phytagel or it will clump. Also, phytagel cannot be re-melted like agar) 0.5ml of 5 mg/ml 2,4-D stock solution (add after autoclaving)

This method produces callus from a much higher percentage of starting seeds faster than the whole seed method. We use this method to initiate callus from Bd21-3.

1) Select immature seeds when most of the seeds are just starting to fill out. Harvest seed heads into 15ml falcon tubes and move to lab. Cap the tubes to keep seeds from drying out.

2) Remove lemma using fingers (the palea adheres too tightly to be removed without damage).

3) Surface sterilize by soaking in a solution of 10% bleach plus 0.1% triton X-100 for 4 minutes. Gently mix tubes while soaking.

4) Rinse 3 times in sterile water in the sterile hood.

5) Dissect embryos out of immature seeds using fine forceps and a dissecting microscope in a laminar flow hood. Smaller embryos work best and large embryos (>0.7 mm) work very poorly. You can expect >90% of small (<0.3 mm) embryos to produce embryogenic callus and >50% of medium sized (0.3-0.7 mm) embryos to form callus. A little practice is required to find the small embryos as they are clear. Older embryos are large and white. See figure 1A.

6) Place embryos on CIM scutellar side down and incubate at 28^{o}C in the dark. Seal plates with parafilm.

7) Callus produced from excised Bd21-3 embryos displays a number of distinct morphologies. First, the embryos start to from an amorphous whitish soft callus. After 1-2 weeks on CIM yellowish callus with organized structures sometimes interspersed with amorphous white callus begins to form. After 3-4 weeks the yellowish callus makes up greater than half of the callus volume (Fig 1 b). The yellowish organized callus and the callus with yellowish organized structures interspersed with amorphous callus both regenerate and are suitable for transformation. At the first subculture it is important to select only yellowish callus to transfer. At the second subculture yellowish callus is again selectively transferred. Probably the best way to get a feel for the system is to put some callus on regeneration media to test it. You can also look at some of the callus under a microscope to verify what you see with the naked eye.

8) Increase callus by subculturing at least every 2 weeks. Don't subculture more than a few times because regeneration becomes a problem with extended time in culture. The regenerability of Bd21-3 seems to decline over time so we try to transform it as soon after callus initiation as we can. Our typical subculturing routine is:

dissect embryos → subculture → subculture → transform
3-4 wks 2 wks 1 wk

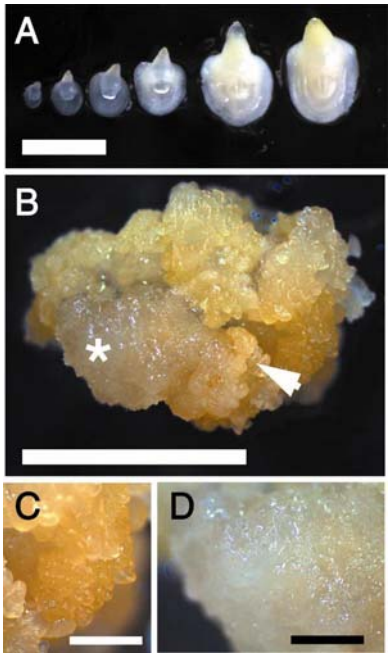

Figure 1. Embryogenic callus initiation. (A) Immature embryos on CI medium. The four embryos on the left are small enough to produce good embryogenic callus and the two large embryos on the right are too mature. Scale 1 mm. (B) Embryo after 3 weeks on CI medium. The yellowish organized callus (arrow) is picked for subculture. Unorganized white, watery callus (asterisk) is discarded. Scale 5 mm. (D) Closeup of embryogenic callus. (E) Closeup of non-embryogenic callus. Scale in D and E is 2 mm.

Transformation starting with embryogenic callus

Materials needed:
antibiotic stock solutions
150 mg/ml timentin solution in water (filter sterilize)
200 mM acetosyringone stock solution
prepare by dissolving 0.392 g in 10 ml DMSO and filter sterilizing (use polypropylene filters). Aliquot and freeze to avoid repeated freeze-thaw cycles.
22.5 mM (5 mg/ml) 2,4-D stock solution (2000 X)
dissolve 50 mg 2,4-D in 10 ml 95% ethanol
0.6 mg/ml $CuSO_4$ (1000 X stock)
9.3 mM (0.2 mg/ml) kinetin stock solution (1000 X)
(dilute from 20 mg/ml stock in DMSO and filter sterilize with nylon filter)
10% Synperonic PE/F68 (Sigma, old name Pluronic F68) filter sterilize

MG/L media per L:
5 g tryptone
2.5g yeast extract
5g NaCl
5 g mannitol
0.1 g $MgSO_4$
0.25 g K_2HPO_4
1.2 g glutamic acid
pH to 7.2 with 1N NaOH
for plates add 15 g agar

Callus initiation media (CIM) per L:
4.43 g LS salts
30 g sucrose
1 ml 0.6 mg/ml $CuSO_4$
pH 5.8 with 0.1 N KOH
2g phytagel for plates (be sure bottles are dry before adding phytagel or it will clump. Also, phytagel cannot be re-melted like agar)
0.5ml of 5 mg/ml 2,4-D stock solution (add after autoclaving)

Regeneration media per L:
4.43 g LS salts
30 g maltose
pH 5.8 with 0.1 N KOH
2 g Phytagel
1 ml of 0.2 mg/ml Kinetin stock solution (added after autoclaving)

MS media per L:
4.42 g MS salts plus vitamins
30 g sucrose
pH 5.7 with 0.1 N KOH
2 g Phytagel

1) One week prior to co-cultivation subculture small pieces (2 mm) callus to fresh CIM and incubate at 28^{o}C.

2) Two days prior to co-cultivation streak out *Agrobacterium* strain from frozen perm on solid MG/L with appropriate antibiotics and incubate at 28-30^{o}. You do not want single colonies and you do not need to add acetosyringone.

3) Prepare the Agro suspension by scraping Agro off the plates and resuspending in liquid CIM to OD_{600} = 0.6. An easy way to do this is to add an excess of Agro, check the OD and dilute as necessary. Add acetosyringone stock solution so the Agro suspension has a final concentration of 200 μM. Add 10uL 10% Synperonic PE/F68 (Sigma, old name Pluronic F68) per 1 mL inoculation media. One plate of Agro should make at least 20 ml of suspension.

4) Immediately transfer the callus pieces from the plates to tubes and add an excess of *Agrobacterium* suspension. Six plates of callus fit easily in a 15 ml Falcon tube and 10 ml of suspension is sufficient. Incubate the callus in the Agro suspension for 5 minutes. I don't think this time is at all critical.

5) Place 1 sterile Whatman filter paper 7cm in a 100 X 15 mm petri plate to the center of the filter paper.

6) Pour off the inoculation media and dump calluses into large petri dish. Carefully suck off excess liquid with 1 mL pipette. Remove as much liquid as possible.

7) Place a reasonable size clump of calluses into each petri dish with filter paper. We typically place all the callus from 2 plates (50 callus pieces) of starting callus on one filter paper (Fig 2A). Spread the calluses around to lightly cover the filter paper.

8) Incubate in dark at 22^{o}C for 3 days.

9) Transfer callus pieces to CIM containing 150 mg/l Timentin (to kill Agro) and appropriate selective agent to kill untransformed plant tissue.** Incubate in dark at 28^{o} for 1 week. The calli recover quickly and grow rapidly.

10) Transfer callus pieces to fresh CIM containing 150 mg/l Timentin (to kill Agro) and appropriate selective agent to kill untransformed plant tissue.** Incubate in dark at 28^{o} for 2 weeks.

11) Continue to subculture and bulk up healthy callus every 2 weeks until you have a few healthy pieces for regeneration. You want to get them onto regeneration media as soon as possible. We typically start to move callus onto regeneration media after 3 weeks on selection and finish moving callus onto regeneration after 5 weeks. Keep callus lines separate so that you can ensure transformants are independent.

12) Transfer healthy callus to regeneration media containing selective agent and incubate in the light (we use 16 hr light 8 hr dark cycle) at 28^{o}. Shoots should appear in 2-4 weeks.

10) When plantlets are large enough to handle safely, transfer and separate plantlets to sundae cups containing MS sucrose for continued growth. (Note, the sundae cups from Solo are inexpensive cups are used for food and although not guaranteed sterile, we have not observed any contamination. If you are planning to do a lot of transformations you might want to try something like these.)

11) When plantlets are large enough, carefully transplant to soil and slowly acclimate to outside conditions. We cover the plants with a plastic dome in a growth chamber (20hr light 4 hr dark). Wait until new growth is obvious, usually about a week, before removing the dome moving to normal growth conditions. Even rootless plantlets have a high survival rate.

*Agro is typically grown with two antibiotics, one for the helper plasmid in the Agro strain and one for the binary plasmid you put in. For derivatives of:

strain AGL1 use 100 mg/L carbinicillin

plus for the following vectors:

pWBVec8 use 25 mg/L spectinomycin liquid and 50 mg/ L for plates

pCT914-1A and pJJ86 use 10 mg/L kanamycin for liquid and 50 mg/ L for plates

** For pWBVec8 and pJJ86 derivatives use 40 mg/L hygromycin (note hygromycin is unstable in water so don't use old stock solutions.)

General notes:
Seal all plates with parafilm.

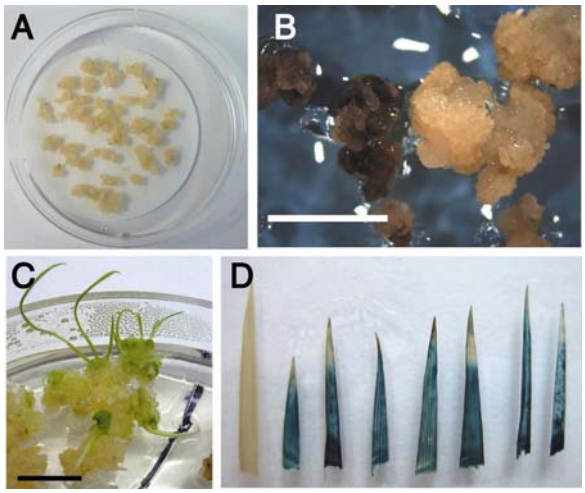

Figure 2. Stages of transformation. (A) Co-cultivation on filter paper. Approximately 50 callus pieces are placed on each filter. The filter paper is 7 cm in diameter. (B) Selection of hygromycin resistant callus. Scale 5 mm. (C) After 2-4 weeks on regeneration media some of the callus produces shoots. Scale 10 mm. (D) GUS stained leaves of seven independent transgenic lines. The non-transgenic control is on the left.

Summary of Brachypodium growth conditions

Callus subculturing and selection

Growth chamber at 28 ^{o}C, dark

T_0 plant regeneration and rooting

Growth chamber (28 ^{o}C, 16-20 hours light / 8-4 hours dark)
(Note: for rooting we use inexpensive 'sundae cups' (Solo Cup Company) instead of tissue culture boxes.)

T_0 plant growth

Growth chamber
20 hr light : 4 hr dark photoperiod, 24 ^{o}C during the day and 18 ^{o}C at night
Cool-white fluorescent lighting at a level of 150 mEm-2s-1
Cover plants with clear plastic dome for the first 3 days
No vernalization required for flowering

Greenhouse
no shading
24 ^{o}C in the day and 18 ^{o}C at night; supplemental lighting to extend day length to 16 hours
requires 2 weeks vernalization at 4 ^{o}C to induce flowering

T_1 plant growth

Growth chamber 22 ^{o}C, cycle 20 hours light/4 hours dark
1 week stratification at 4 ^{o}C
Greenhouse: requires 3 weeks vernalization at 4 ^{o}C for flowering