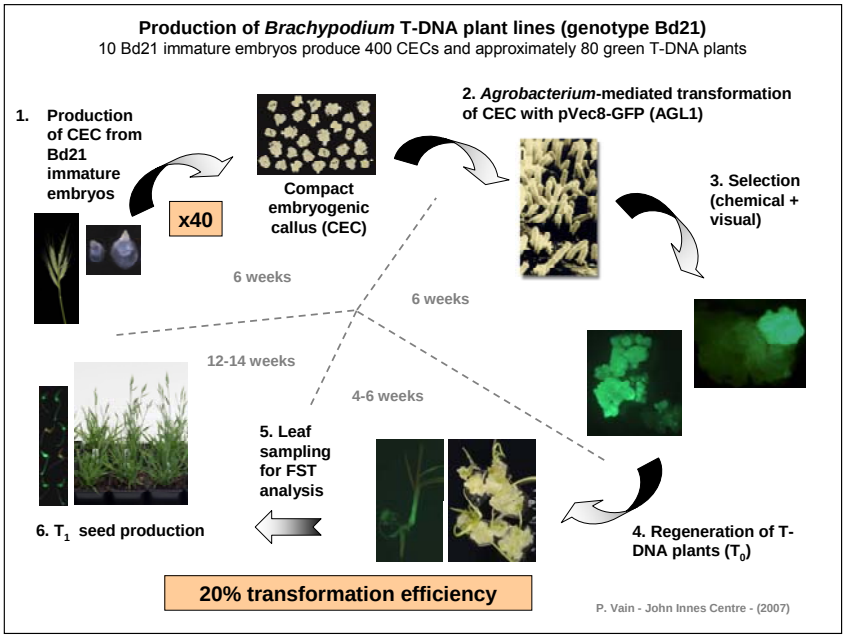
BrachyTAG

Protocol for *Brachypodium* T-DNA plant production

Vain P. et al. (2008) Plant Biotechnology Journal, 6:236-245

Alves S.C. et al. (2009) Nature Protocols, 4:638-649.



Production of compact embryogenic callus from immature embryos

1. Collect tillers from 7-9 weeks-old Bd21 plants when the immature seeds are swollen but still green.
2. Select immature seeds with a soft endosperm, remove the top glume (lemma) from seeds. Collect seeds in water. Drain well before sterilisation.
3. Sterilise approximately 20 seeds for 30 sec with 20 ml of 70% ethanol in a sterile Petri dish with a lid. Drain ethanol and rinse with sterile deionised water. Add 20 ml of 1.3% sodium hypochlorite solution. Gently shake the seeds for 4 min. Rinse three times with sterile deionised water.
4. Isolate immature embryos up to and including 0.3 mm in length from seeds. Only very small immature translucent embryos will produce homogeneous CEC at high frequency.



5. Culture immature embryos, scutellum facing up, onto MSB3+Cu0.6 solid medium (MS salts, Fe-EDTA, 30 g/l sucrose, 2.5 mg/l 2,4-D, 0.6 mg/l $CuSO_4$, vitamins M5, 2 g/l Phytagel - pH 5.8) for 3 weeks at 25°C in the dark. $CuSO_4$ significantly promotes the growth and embryogenicity of Bd21 CEC.
6. Excise the shoots under sterile conditions, as they elongate during the first 2-3 days of culture.
7. At week 3, fragment CEC with a creamy colour and pearly surface in 1-3 pieces. Transfer pieces of CEC onto fresh MSB3+Cu0.6 solid medium for another 2 weeks at 25°C in the dark. Discard all non-CEC tissue.
8. At week 5, split CEC with a creamy colour and pearly surface in 4-6 pieces. Transfer pieces of CEC onto fresh MSB3+Cu0.6 solid medium for another week at 25°C in the dark. Discard all non-CEC tissue.
9. At week 6 (on the day of transformation), split CEC one last time in 4-6 pieces and place 50-100 CEC pieces on fresh MSB3+Cu0.6 solid medium before inoculation with *Agrobacterium*.

Preparation of *Agrobacterium* inoculum

10. Suspend 5 µl of *Agrobacterium tumefaciens* (AGL1 strain), carrying the pVec8-GFP binary vector, from a glycerol stock stored at -80°C, into 1 ml of LB liquid medium supplemented with 50 mg/l streptomycin.
11. Grow overnight in an incubator-shaker at 28°C and 200 rpm.
12. Plate 200 µl of the overnight culture onto solid MGL medium supplemented with 50 mg/l streptomycin and 30 mg/l acetosyringone using a sterile spreader. Culture plates (upside down) for 2 days at 28°C in the dark.

***Agrobacterium*-mediated transformation of compact embryogenic callus**

13. Scrape *Agrobacterium* layer with a L-shaped sterile glass Pasteur pipette and add to a 50-ml disposable sterile tube containing 10 ml of MSB+AS45 liquid medium (MS salts, Fe-EDTA, 10 g/l sucrose, 10 g/l mannitol, 45 mg/l acetosyringone – pH 5.5). Strongly shake tube by hand to suspend *Agrobacterium*.
14. Shake suspension at 220 rpm for 45 min in a 28°C incubator to continue dispersing *Agrobacterium*.



15. Measure optical density of the suspension at λ=600 nm and dilute to $O.D._{600}$=1 using MSB+AS45 liquid medium.
16. Flood CEC plates with 13 ml of *Agrobacterium* suspension and leave for a 5-min inoculation in a laminar flow hood at room temperature.
17. Pipette out the bacterial suspension completely from the CEC plates and handpick each callus piece straight onto a dry sterile filter paper placed in an empty Petri dish. Leave CECs uncovered under the laminar flow hood for 7 min as a desiccation treatment.
18. Co-culture CECs from on MSB3+AS60 medium (MS salts, Fe-EDTA, 30 g/l sucrose, 2.5 mg/l 2,4-D, vitamins M5, 2 g/l Phytagel, 60 mg/l acetosyringone - pH 5.8) plates for 2 days at 25ºC in the dark.

17-18 Alternative steps: Co-culture can also be conducted on a filter paper instead of a jellified medium. In step 17, pipette out the bacterial suspension, handpick CECs, blot excess inoculum by a quick touch on a dry sterile filter paper (omit the 7-min desiccation treatment) and transfer onto a 9-cm filter paper wetted with 750 µl of MSB+AS45 medium in an empty Petri dish. Incubate for 2 days at 25ºC in the dark.

Selection of transformed calli

19. Transfer co-cultured CECs onto MSB3+Cu0.6+H40+T225 solid medium (MS salts, Fe-EDTA, 30 g/l sucrose, 2.5 mg/l 2,4-D, 0.6 mg/l $CuSO_4$, vitamins M5, 2 g/l Phytagel, 225 mg/l timentin and 40 mg/l hygromycin B - pH 5.8). Culture for 3 weeks at 25ºC in the dark.
20. Three weeks after transformation, screen CEC growing on hygromycin for the presence of small bright green fluorescent (GFP) sectors. Dissect each fluorescent sector and culture them as independent T-DNA lines onto MSB3+Cu0.6+H30+T225 solid medium (MS salts, Fe-EDTA, 30 g/l sucrose, 2.5 mg/l 2,4-D, 0.6 mg/l $CuSO_4$, vitamins M5, 2 g/l Phytagel, 225 mg/l timentin and 30 mg/l hygromycin B - pH 5.8) for another 3 weeks at 25ºC in the dark.

Regeneration of T-DNA plants

21. Six weeks after transformation, screen calli for green fluorescence using a stereomicroscope with a UV light source. Transfer hygromycin-resistant and GFP+ calli onto the MSR26+H20+T225 regeneration medium (MS salts, Fe-EDTA, 30 g/l sucrose, 0.2 mg/l kinetin, vitamins M5, 2 g/l Phytagel, 225 mg/l timentin and 20 mg/l hygromycin B - pH 5.8) for

2-3 weeks at 25ºC under 16-h photoperiod. Keep all GFP+ callus pieces from the same independent T-DNA line together for regeneration.

22. Eight to nine weeks after transformation, transfer shoots (rooted or not) to tubes containing MSR63+Ch7+T112 germination medium (40% MS salts, Fe-EDTA, 10 g/l sucrose, vitamins B5, 7 g/l charcoal, 2 g/l Phytagel, 6 g/l agar2, 112 mg/l timentin - pH 5.8). Culture for 2-3 weeks at 25ºC under 16-h photoperiod.

23. Ten-eleven weeks after transformation, confirm that plantlets in tubes are GFP+ before transferring to CER. GFP fluorescence is easily detected in the roots of plantlets within the tubes using a stereo microscope equipped with a fluorescent module.

Seed production from T-DNA plants

1. Transfer fully rooted T-DNA plantlets in 2x2 cm cell-tray containing a wet compost mixture. Grow plants in a Controlled Environment Room (CER) at 22°C with a 20-hour photoperiod. Initially, keep seedlings covered with a propagator lid for 1-2 weeks to ensure adaptation of plantlets to low humidity conditions.
2. Transfer compost and plant (when starting to tiller) into “Rootrainer” pots containing a wet compost mixture. Grow plants in a CER at 22°C with a 20-hour photoperiod for 5-6 weeks.
3. When the seeds are fully mature, stop watering plants and dry plants for 2-4 weeks.
4. Collect spikelets from each plant and store at 1.5°C, 7-10% humidity in the dark. Seeds can be stored for more than 10 years in these conditions.

Reagents, compost and culture media composition, as well as additional practical information is detailed in Alves S.C. et al. (2009) Nature Protocols, 4:638-649.