

Biolistic Transformation of Wheat

This method is adapted from (Barcelo and Lazzeri 1995, Pastori *et al.* 2001, Rasco-Gaunt *et al.* 2001, Sparks and Jones 2003).

Growth of donor plants

Wheat (*Triticum aestivum* L.) plants are grown at 18-20°C day and 14-15°C night temperatures under a 16 h photo-period provided by banks of HQI lamps (400W) in growth rooms. Winter wheat varieties are vernalised from seed for 8 weeks at 4-5°C. Initially all plants are top watered but once the root system reaches the base of the pot, the plants are watered using an automated flooding system. Pests and disease are kept to a minimum by good housekeeping practices but (*Amblyseius caliginosus*) is used as a biological control agent to manage thrips, and the fungicide Fortress is applied as a preventative spray to avoid mildew. If plants appear diseased, they are discarded immediately.

Collection and sterilisation of wheat caryopses

Collect spikes from growth room-grown plants at ~10-12 weeks after sowing. Embryos at the correct stage are usually found ~12-16 days post anthesis. Remove the caryopses from the panicles and sterilise by rinsing in 70% (v/v) aqueous ethanol for 5 min then soak for 15-20 min in 10% (v/v) Domestos with gentle shaking on a platform shaker (~ 60 rpm). Rinse copiously with at least three changes of sterile water.

Isolation and pre-culture of immature scutella

Isolate the embryos microscopically in a sterile environment and remove the embryo axis to prevent precocious germination. Embryos of size approximately 0.5 - 1.5 mm are generally most responsive but there is genotypic variation. Place 25-30 scutella per 9 cm Petri-dish containing induction medium (MS9%0.5D), locating them within a central target area and orientating them

with the uncut scutellum uppermost i.e. the uncut side is bombarded (see Fig. 1B). 1 plate, ~15 embryos, should be prepared as a control. This will be bombarded but with gold without any DNA. Pre-culture the prepared donor material in the dark at 26°C for 1-2 days prior to bombardment.

Preparation of gold particles

Place 20 mg BIO-RAD sub-micron gold particles (0.6 μm) and 1ml 100% ethanol into a 1.5 ml eppendorf. Sonicate for 2 min, pulse spin in a microfuge for 3 sec and remove the supernatant. Repeat this ethanol wash twice more. Add 1 ml sterile water, sonicate for 2 min, pulse spin in a microfuge for 3 sec and remove the supernatant. Repeat this step. Resuspend gold in 1 ml sterile water, divide into 50 μl aliquots and store at -20°C. When ready to precipitate DNA onto gold, allow a 50 μl aliquot of prepared gold to thaw at room temperature then sonicate for 1-2 min. Add 5 μl DNA (1 mg/ml in TE or water) or water for bombarded-only controls and vortex briefly. Place 50 μl 2.5M CaCl_2 and 20 μl 0.1M spermidine into the lid of the eppendorf and vortex into the gold + DNA solution. Pellet the DNA-coated particles by centrifugation and discard the supernatant. Add 150 μl 100% ethanol to wash the particles and re-suspend. Again, pellet the particles and discard the supernatant. Resuspend fully in 85 μl 100% ethanol and maintain on ice.

Delivery of DNA-coated gold particles

Note: The PDS-1000/He particle gun [BIO-RAD] delivery system involves the use of high pressure gas to accelerate particles to high velocity. Appropriate safety precautions described by the manufacturer should be taken when operating the gun.

The following settings are recommended for this procedure: gap 2.5 cm, stopping plate aperture 0.8 cm, target distance 5.5 cm, vacuum 91.4 – 94.8 kPa, vacuum flow rate 5.0, vent flow rate 4.5. Sterilise the gun's chamber and component parts with 90% (v/v) ethanol. Sterilise macro-carrier holders, macro-carriers, stopping screens and rupture discs by dipping in 100% ethanol and

allow to evaporate completely. Briefly vortex the coated gold particles, take 5 μ l, place centrally onto the macro-carrier membrane and allow to dry. Load a rupture disc (650 or 950 psi) into the rupture disc retaining cap and tightening the screw into place firmly. Place a stopping screen into the fixed nest. Invert the macro-carrier holder containing macro-carrier + gold particles/DNA and place over the stopping screen in the nest and maintain its position using the retaining ring. Mount the fixed nest assembly onto the second shelf from the top to give a gap of 2.5 cm. Place a sample on the target stage, draw a vacuum of 91.4 - 94.8 kPa and fire the gun.

Tissue culture and the selection of transformants

Following bombardment, treated explants are distributed over induction medium, at ten per 90-mm Petri dish. Cultures are incubated at 26°C in darkness for 3 weeks. Embryogenic calluses are subsequently transferred to R or RZD regeneration medium for shoot induction. Regenerating calluses are cultured at 26°C in the light, and subcultured on to fresh, hormone-free R medium every three weeks. Selection is started in either the first or second round of regeneration and continued in subsequent rounds.

The selection agent is dependent upon the selectable marker in the transforming plasmid. Plantlets surviving three to four rounds of selection should have established a reasonable root system and be outgrowing the magentas. At this stage, about 3 months after bombardment, the plantlets should be transferred to soil. It is better, initially, to plant them into small pots and under a propagator for 1-2 weeks to acclimatize them after tissue culture. Once established the plants are re-potted into 13 cm pots and grown to maturity under normal glasshouse conditions. This will take a further 3 – 4 months.

Transgenic plants can be analysed in a number of ways: Marker gene expression can be assessed using for example, UV visualisation of GFP, the histochemical GUS test for *uidA* and herbicide leaf paint assay (1 and/or the ammonium test for *bar*). Gene integrations can be determined using PCR, Southern analysis and fluorescent in situ hybridisation (FISH)

Media

Induction media

MSS 3AA/2 9%S (x2):- 200 ml/l MS macrosalts, 2 ml/l L7 microsals, 20 ml/l Ferrous sulphate chelate solution (x100) (Sigma-Aldrich), 2 ml/l MS (-Glycine) vitamins, 200 mg/l myo-Inositol (Sigma-Aldrich), 40 ml/l 3AA amino acids, 180 g/l (9% final concentration) sucrose (Sigma-Aldrich). pH adjusted to 5.7 with 5M NaOH or KOH. Osmolarity should be within the range 800-1100 mOsM. Filter sterilise and store at 4⁰C .

MS9%0.5D:- Mix an equal volume of MSS 3AA/2 9%S (x2) with sterilised, melted agar. Add 0.5 mg/l 2,4-D and 10 mg/l AgNO₃ and pour into 9 cm diameter Petri-dishes (Bibby Sterilin Ltd., Staffordshire, UK) (~28 ml per dish). Store at 4⁰C in the dark.

Regeneration media

R (x2):- 200 ml/l L7 macrosalts, 2 ml/l L7 microsals, 20 ml/l Ferrous sulphate chelate solution (x100), 10 ml/l L7 vitamins/inositol, 60 g/l maltose (Melford Laboratories Ltd.). Adjust pH to 5.7 with 5M NaOH or KOH. Osmolarity should be within the range 269-298 mOsM. Filter sterilise and store at 4⁰C .

RZD: Mix an equal volume R (x2) with sterilised, melted agar. Add 5 mg/l zeatin, 0.1 mg/l 2,4-D and 10 mg/l AgNO₃ and pour into 9 cm Petri-dishes (~28 ml per dish). Store at 4⁰C in the dark.

Selection media:

N.B. Dependent on selectable marker in transformation plasmid.

Concentration of selection agent also variable, development of cultures should be taken into account at time of transfers. Generally use within the range 2-4 mg/l PPT and 25-50 mg/l G418

RZDPPT4 or RZDG50: Mix an equal volume of R (x2) with sterilised, melted agar and add 5 mg/l zeatin, 0.1 mg/l 2,4-D and 4 mg/l Glufosinate ammonium (PPT4) or 50 mg/l G418 (G50). Pour into 9 cm Petri-dishes (~28 ml per dish) or GA-7 Magenta vessels (Sigma-Aldrich) (~60 ml per vessel). Store at 4°C.

RPPT4 or RG50: Mix an equal volume of R (x2) with sterilised, melted agar and add 4 mg/l Glufosinate ammonium (PPT4) or 50 mg/l G418 (G50). Pour into 9 cm Petri-dishes (~28 ml per dish) or GA-7 Magenta vessels (~60 ml per vessel). Store at 4°C.

Bombardment materials

2.5M Calcium chloride (Fisher Scientific UK): Dissolve 3.67 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in 10 ml water. Mix well/vortex. Filter sterilise and store at -20°C in 50 µl aliquots.

0.1M Spermidine free-base (Sigma-Aldrich): Prepare 1M stock from powder in sterile water and maintain at -80°C in 20 µl aliquots. Prepare the 0.1M working solution by making a 1:10 dilution of 1M stock in sterile water under sterile conditions. Mix well, aliquot in 10 µl volumes and store immediately at -20°C .

References

Barcelo P and Lazzeri PA. 1995. Transformation of cereals by microprojectile bombardment of immature inflorescence and scutellum tissues. In: Methods in Molecular Biology. Vol 49: Plant Gene Transfer and Expression Protocols, H.Jones (Ed). Humana press, Totowa.

Pastori G, Wilkinson M, Steele S, Sparks C, Jones HD and Parry MAJ (2001). Transformation of elite wheat cultivars at high frequencies. *J. Exp. Bot.* 52(357): 857-863.

Rasco-Gaunt S, Riley A, Cannell M, Barcelo P, Lazzeri PA. 2001. Procedures allowing the transformation of a range of European elite wheat (*Triticum aestivum* L.) varieties *via* particle bombardment. *J. Exp Bot.* 52(357):865-874.

Caroline Sparks and Huw D Jones (2003). Transformation of Wheat by Biolistics. In; Transgenic Crops of the World. Ed. Ian Curtis. Kluwer Academic Publishers (In press)