Agrobacterium-mediated transformation of soybean and recovery of transgenic soybean plants

Paz et al. (2005) Improved cotyledonary node method using an alternative explant derived from mature seed for efficient *Agrobacterium*-mediated soybean transformation. Plant Cell Reports 2005.

Materials

⌘ Plasmid

Different plant transformation constructs that were derivatives of base vector pTF101.1 were introduced into *Agrobacterium tumefaciens* strain EHA101 (Hood et al. 1986). The base vector pTF101.1 is a derivative of the pPZP binary vector (Hajdukiewicz et al., 1994) that includes the right and left T-DNA border fragments from a nopaline strain of *A. tumefaciens,* a broad host origin of replication (pVS1) and a spectinomycin-resistant marker gene (*aadA*) for bacterial selection. The plant selectable marker gene cassette consists of (1) double 35S promoter (2x P35S) of cauliflower mosaic virus (CaMV) (Odell et al. 1985), (2) tobacco etch virus translational enhancer (Carrington and Freed 1990), (3) the phosphinothricin acetyl transferase (*bar)* gene from *Streptomyces hygroscopicus* that confers resistance to the herbicide phosphinothricin and its derivatives.). pTF101.1 contains a multiple cloning site (MCS) for facilitating subcloning of the gene of interest in between the right border region and the plant selectable marker cassette. The soybean vegetative storage protein terminator (Mason et al., 1993) was cloned to the 3' end of the *bar* gene. The vector pTF102 was derived from pTF101.1 by inserting the P35S GUS intron cassette (Vancanneyt et al. 1990) into the *Hin*d III site of pTF101.1 The *gus* gene contained a portable intron in its codon region (Vancanneyt et al., 1990) to prevent GUS activity in *Agrobacterium* cells.

⌘ Plant material

Soybean cultivars Thorne, Williams, Williams79, and Williams82

Media

⌘ YEP Solid Medium:

5 g/L Yeast extract, 10 g/L Peptone, 5 g/L $NaCl_2$, 12 g/L Bacto-agar. pH to 7.0 with NaOH. Appropriate antibiotics should be added to the medium after autoclaving. Pour into sterile 100x15 plates (~25ml per plate).

⌘ YEP Liquid Medium:

5 g/L Yeast extract, 10 g/L Peptone, 5 g/L $NaCl_2$. pH to 7.0 with NaOH. Appropriate antibiotics should be added to the medium prior to inoculation.

⌘ Co-cultivation Medium:

1/10X B5 major salts, 1/10X B5 minor salts, 2.8 mg/L Ferrous, 3.8 mg/L NaEDTA, 30 g/L Sucrose, 3.9 g/L MES, and 4.25 g/L Noble agar (pH 5.4). Filter sterilized 1X B5 vitamins, GA_3 (0.25 mg/L), BAP (1.67 mg/L), Cysteine (400 mg/L), Dithiothrietol (154.2 mg/L), and 40 mg/L acetosyringone are added to this medium after autoclaving. Pour into sterile 100x15 mm plates (~88 plates/L). When solidified, overlay the co-cultivation medium with sterile filter paper to reduce bacterial overgrowth during co-cultivation (Whatman #1, 70 mm).

⌘ Infection Medium:

1/10X B5 major salts, 1/10X B5 minor salts, 2.8 mg/L Ferrous, 3.8 mg/L NaEDTA, 30 g/L Sucrose, 3.9 g/L MES (pH 5.4). Filter sterilized 1X B5 vitamins, GA_3 (0.25 mg/L), BAP (1.67 mg/L), and 40 mg/L acetosyringone are added to this medium after autoclaving.

⌘ Shoot Induction Washing Medium:

1X B5 major salts, 1X B5 minor salts, 28 mg/L Ferrous, 38 mg/L NaEDTA, 30 g/L Sucrose, and 0.59 g/L MES (pH 5.7). Filter sterilized 1X B5 vitamins, BAP (1.11 mg/L), Timentin (100 mg/L), Cefotaxime (200 mg/L), and Vancomycin (50 mg/L) are added to this medium after autoclaving.

⌘ Shoot Induction Medium I:

1X B5 major salts, 1X B5 minor salts, 28 mg/L Ferrous, 38 mg/L NaEDTA, 30 g/L Sucrose, 0.59 g/L MES, and 7 g/L Noble agar (pH 5.7). Filter sterilized 1X B5 vitamins, BAP (1.11 mg/L), Timentin (50 mg/L), Cefotaxime (200 mg/L), and Vancomycin (50 mg/L) are added to this medium after autoclaving. Pour into sterile 100x20 mm plates (26 plates/L).

⌘ Shoot Induction Medium II:

1X B5 major salts, 1X B5 minor salts, 28 mg/L Ferrous, 38 mg/L NaEDTA, 30 g/L Sucrose, 0.59 g/L MES, and 7 g/L Noble agar (pH 5.7). Filter sterilized 1X B5 vitamins, BAP (1.11 mg/L), Timentin (50 mg/L), Cefotaxime (200 mg/L), Vancomycin (50 mg/L) and Glufosinate (6 mg/L) are added to this medium after autoclaving. Pour into sterile 100x20 mm plates (26 plates/L).

⌘ Shoot Elongation Medium:

1X MS major salts, 1X MS minor salts, 28 mg/L Ferrous, 38 mg/L NaEDTA, 30 g/L Sucrose, 0.59 g/L MES, and 7 g/L Noble agar (pH 5.7). Filter sterilized 1X B5 vitamins, Asparagine (50 mg/L), L-Pyroglutamic Acid (100 mg/L), IAA (0.1 mg/L), GA_3 (0.5 mg/L), Zeatin-R (1 mg/L), Timentin (50 mg/L), Cefotaxime (200 mg/L), Vancomycin (50 mg/L), and Glufosinate (6 mg/L) are added to this medium after autoclaving. Pour into sterile 100x25 mm plates (22 plates/L).

⌘ Rooting Medium:

1X MS major salts, 1X MS minor salts, 28 mg/L Ferrous, 38 mg/L NaEDTA, 20 g/L Sucrose, 0.59 g/L MES, and 7 g/L Noble agar (pH 5.6). Filter sterilized 1X B5 vitamins, Asparagine (50 mg/L), and L-Pyroglutamic Acid (100 mg/L) are added to this medium after autoclaving. Pour into sterile 150x25 mm vial (10ml/vial).

Methods

⌘ Seed Sterilization

1. Place mature soybean seeds in 100x15 mm petri plates in a single layer (about 130 seeds per plate).

2. Arrange 3-4 plates in a bell jar desiccator within a fume hood in such a way that all interior plate surfaces are exposed and allow enough space to accommodate a 250 ml beaker.

3. Using appropriate hand protection, fill the 250 ml beaker with 100ml of bleach and add 3.5 ml of concentrated (12N) HCl drop wise along the side of the beaker.

4. Close the desiccator immediately and let stand overnight (16 hours).

5. After overnight exposure to chlorine gas, close the petri plates and remove them to a laminar flow hood. Open the plates and allow them to air out for about 30 minutes to remove the excessive chlorine gas.

⌘ *Agrobacterium* Preparation

1. Bacteria cultures for weekly experiments are initiated from –80ºC glycerol stocks three days prior to an experiment. The vector system, pTF102 in EHA101, is cultured on YEP medium (An et al., 1988) containing 100 mg/L spectinomycin (for pTF102), 50 mg/L kanamycin (for EHA101), and 25 mg/L chloramphenicol (for EHA101).

2. 24 hours prior to the experiment start a 2 ml culture of *Agrobacterium* by inoculating a loop of bacteria from the fresh YEP plate in YEP liquid medium amended with antibiotics.

3. Allow the culture to grow to saturation (8-10 hours) at 28^{o}C in a shaker incubator (~250 rpm). At the end of the day, transfer 0.2 ml of starter culture to a 1 L flask containing 250 ml of YEP medium amended with antibiotics.

4. Allow the culture to grow overnight at 28°C, 250 rpm to log phase (OD_{650} = 0.3 – 0.6 for EHA105) or late log phase (OD_{650} = 1.0 – 1.2 for EHA101).

5. Collect *Agrobacterium* culture by pelleting at 3,500 rpm for 10 minutes at 20°C.

6. Resuspend the pellets in infection medium by pipetting through the pellet. Bacterial cell densities are adjusted to a final OD_{650}=0.6 (for EHA105) or OD_{650}=0.6 to 1.0 (for EHA101).

7. Gently shake the resulting infection medium at 60 rpm for at least 30 minutes before use.

⌘ Seed Imbibition

1. Under the laminar flow hood, approximately 20 hours prior to the experiment, add deionized sterile water to the sterilized seeds until the water is ¼ cm from the top of the plate.

2. Completely cover plate with aluminum foil to block out light.

⌘ Explant Preparation and Infection

1. Remove aluminum foil from the imbibed soybean seeds. Transfer ~20 seeds to a sterile 100x15 petri plate for dissection.

2. Using a #15 scalpel blade, make a longitudinal cut along the hilum to separate the cotyledons and remove the seed coat. Excise the embryonic axis found at the nodal end of the cotyledons, and remove any remaining axial shoots/buds attached to the cotyledonary node.

3. Dissect 60 half-seed explants (30 seeds) into a 100 x 25 mm petri plate and add 30 ml of *Agrobacterium* infection media. Make sure the explants are completely covered by the infection media. Allow the explants to incubate at room temperature for 30 minutes with occasional gentle agitation.

⌘ Co-Cultivation

1. After infection, remove excess infection media by gently grasping the explant with sterile forceps and tapping the forceps on the rim of the infection media plate. Transfer half-seed explants to co-cultivation medium (6 per plate) so the flat, adaxial side is touching the filter paper.

2. Wrap the plates with parafilm and place them at 24^{o}C under an 18:6 photoperiod (140 µmoles s^{-1} m^{-2}) for 5 days.

⌘ Shoot induction

1. After five days of co-cultivation, briefly wash the half-seed explants in shoot induction washing medium (~50ml in a 100 x 25 sterile petri plate, room temperature). 30 half-seed explants may be washed for each plate of washing medium.

2. Place the explants on shoot induction medium I (5 explants per plate). Half-seed explants should be oriented with the nodal end of the cotyledon imbedded in the medium and the regeneration region flush to the surface with flat side up at a 30–45^{o} angle.

3. Wrap each plate with vent tape and incubate at 24^{o}C, 18:6 photoperiod for 14 days (if plates are stacked, by the end of the first week, rearrange each plate in each stack so that the top and bottom plates are switched and explants are exposed to light).

4. Explants should be transferred to shoot induction medium II after 14 days. Cut and discard large shoots and make a fresh cut at the base of the shoot pad flush to the medium. Orient the tissue in such a way that the freshly cut surface is imbedded into the fresh shoot induction medium, with the differentiating region flush to the surface.

5. Maintain cultures in the Percival incubator under the same conditions described above for another 14 days.

⌘ Shoot Elongation

1. After 4 weeks on shoot induction medium, remove the cotyledons from the explants and make a fresh cut at the base of the shoot pad flush to the medium.

2. Transfer the explants to fresh shoot elongation medium and incubate the tissue at 24°C, 18:6 photoperiod for 2-8 weeks.

3. Transfer the tissue to fresh shoot elongation medium every 2 weeks. At each transfer make a fresh horizontal slice at the base of the shoot pad.

⌘ Rooting of transgenic plants

1. When shoots surviving glufosinate selection reach at least 3 cm, excise them from the shoot pad.

2. Soak the cut end of the shoots in filter sterilized Indole-3-butyric acid (IBA), 1 mg/ml, for 1–2 minutes. Then transfer shoots to rooting medium in 150 x 25 mm glass vials with the stems of the shoots embedded approximately ½ cm into the media.

3. Incubate at 24°C, 18:6 photoperiod for 1-2 weeks.

⌘ Plant Acclimatization and Liberty Screening

1. After 1-2 weeks, when the shoot develops more than two roots, transplant it into soil. Gently remove the plant from the rooting medium and wash off the roots with tap water to remove any excess medium.

2. Soak 2.5 inch jiffy pots in tap water to assist in even hydration. Place each soaked jiffy pot into an individual 2.5 inch plastic pot (cut from a four-pack) and fill with moistened Redi-Earth Peat-Lite soil mix (Hummert Cat. # 10-2030-1). Transplant plantlets to the soil and place them in a flat without holes, covered with a humidome. Allow the plantlets to grow at 24°C, 18:6 photoperiod for at least one week, watering as needed.

3. When the plantlets have at least two healthy trifoliates, an herbicide paint assay may be applied to confirm resistance to glufosinate. Using a cotton swab, apply Liberty herbicide (150mg l^{-1}), to the upper leaf surface along the midrib of two leaves on two different trifoliates. Transfer painted plants to the greenhouse and cover with a humidome. Score plantlets 3-5 days after painting. Resistant plantlets may be transplanted immediately to 2-gallon pots.

4. Fill 50% of a 2-gallon nursery pot with 4 drainage holes with Sunshine Universal Mix SB300. Add 1 (15 g per 2 gal. pot) Sierra 16-8-12 controlled release fertilizer tablet with trace elements to each pot.

5. Add additional Universal Mix to bring the soil volume to 80% of the pot (~2" from top edge of pot). Too little does not hold enough water between watering, and too much does not allow enough water to be added at watering.

6. Transplant Liberty resistant plantlets, including the jiffy pots, to the middle of the 2-gallon pot. The soil should cover all the roots. Be sure to plant the young plants deep enough or they will tip over when they grow taller.

7. Fill with water to the top edge of the pot. Let it drain completely, and water once more until the water reaches the top of the pot. The plants will not need to be watered until 7-14 days after transplant. After this time, water as needed.

⌘ **Plant Care**

1. Marathon, for aphid control, may be added as part of the transplant step during summer months. If white flies or fungus gnats hover over pots, yellow sticky sheets may be used to reduce or eliminate the insects.

2. As plants grow, staking will be required to prevent plants from intertwining. Loosely bind elongated soybean branches to long bamboo stakes using twist ties.

3. Soybean pods on the same plant will dry at variable rates. To prevent pod shatter and consequent seed loss due to over drying, remove dry pods and store them in a paper bag until all pods on the plant are harvested.

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