Protocol

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Stable Maize Transformation Protocol

Adapted from Raizada and Walbot, Stanford University (2001)

Introduction

Biolistic transformation is a widely used method for introducing genes into plants that are resistant to *Agrobacterium*-mediated transformation. This technique promotes gene transfer through direct delivery, resulting in stably transformed maize callus. Here, we present a protocol that details the biolistic transformation of maize embryogenic callus using the pAHC20 plasmid. This plasmid contains the ubiquitin promoter and the *bar* gene, which provides resistance to the herbicide Basta® or bialaphos and serves as a selective marker.

Materials

- A188 x B73
- N6 1-100-25 + $AgNO_3$ Media
- N6 1-100-25 media
- $AgNO_3$
- Osmotic media
- D-mannitol (GoldBio Catalog # M-060)
- M13 DNA
- Maxiprep kit
- Phenol-chloroform
- Spermidine trihydrochloride (GoldBio Catalog # S-715)
- Ice
- Ethanol
- Spherical Gold Particles
- Bialaphos (GoldBio Catalog # B0178)
- Sorbitol

Preparation of N6 1-100-25 media + $AgNO_3$

- 4 g N6 Basal salts
- 500 µl 2 mg/L 2,4-D (final concentration 1 mg/L)
- 100 mg casamino acids (final concentration 100 mg/L)
- 2.88 g L-proline (final concentration 25mM)
- 20 g sucrose (final concentration 20 g/L)
- 1 ml 1000x N6 vitamins (final concentration 1x)
- Adjust pH to 5.8 with 1N KOH and 1N acetic acid

- Add ddH_2O to 1 L
- Add 2 g Phytagel (final concentration 2 g/L) while stirring rapidly or shaking after addition.
- Autoclave for 25 minutes and cool at 47-50°C for 45 minutes.
- Add 1 ml of filter-sterilized 10mM $AgNO_3$ (final concentration 10µM)

Osmotic induction media

- Add 36.4 g Mannitol (0.2M final concentration)
- Add 36.4 g Sorbitol (0.2M final concentration)
- Add N6 1-100-25 media to 1 L.

Method

Establishment of Embryogenic Callus Cultures

1. Obtain an A188 x B73 seed and either sib- or self-pollinate.

2. After 9-13 days of pollination, dissect 1-2 mm embryos in the dark and induce calli on Petri dishes containing N6 1-100-25 + $AgNO_3$ media (subculture induction media).

   Note: Maintain calli in complete darkness at 27°C and 75% RH in 100 x 25 mm Petri dishes during callus induction, bombardment and herbicide selection protocols.

3. After 4 weeks in subculture induction media, transfer calli to media without $AgNO_3$ and maintain for up to 3 months with a 2-week subculture regime that selects for white, embryogenic, friable tissues at each sub culture. Initiate new cultures every 3 months.

Bombardment

In bombardment, first coprecipitate equimolar quantities of plasmids. Then, accelerate DNA/gold particles into immature somatic embryo tissue using the Helium PDS 1000HE device. Bombardment should be performed in sterile conditions and at room temperature. In addition, tissues used for bombardment should be subcultured for at least 5-9 days.

1. Isolate M13 DNA (plasmid and double-stranded) using a Maxiprep Kit.

   Note: Extract DNA once with phenol-chloroform and ethanol as follow:
   - **a. Add an equal volume of phenol-choloroform to DNA.**
   - **b. Vortex the sample to mix for 30 seconds.**
   - **c. Centrifuge sample for 5 minutes at room temperature. After centrifugation there should be 3 separate layers: top (aqueous DNA phase), middle (white layer containing proteins), and bottom (protein).**
   - **d. Recover the DNA (top layer) with a pipet without disturbing the other layers, and place in a new tube.**
   - **e. Add an equal volume of chloroform. Repeat steps b-d.**

f. **Add 2 volumes of 95-100% ethanol. Invert to mix.**
g. **Place the DNA in dry ice for 30 minutes or -20°C overnight to help DNA precipitate.**
h. **Centrifuge DNA at maximum speed (~12,000 rpm) for 15-30 minutes at 4°C.**
i. **Discard the supernatant and ensure liquid is removed completely from the tube without disturbing the pellet.**
j. **Wash the pellet with 500 µl cold 70% ethanol.**
k. **Centrifuge DNA at maximum speed (~12,000 rpm) for 15-30 minutes at room temperature.**
l. **Discard the supernatant and dry the pellet for 5-20 minutes at room temperature.**
m. **Resuspend the pellet with TE and store at 4°C. Continue onto step 5.**

2. For 3 bombardments, centrifuge 2 mg of gold at 2000 rpm in a microcentrifuge tube.

3. Rinse the gold pellet with molecular biology grade water, recentrifuge (2000 rpm) and resuspend in 25 µl of 1 µg/µl total DNA.

4. Add 220 µl molecular biology grade water, 250 µl 2.5M $CaCl_2$ and 50 µl 0.1M Spermidine trihydrochloride to the tube, vortexing between each addition.

5. Place the mixed DNA precipitate on ice for 5 minutes.

6. Vortex for 1-2 minutes at room temperature and centrifuge at 500 rpm for 5 minutes.

7. Remove the supernatant and resuspend the pellet in 600 µl of ethanol.

8. Centrifuge for 1 minute at 14,000 rpm.

9. Resuspend the pellet in 36 µl ethanol. Use immediately or store on ice for up to 4 hours prior to bombardment.

10. About 4-6 hours before bombardment, remove ~5 mm clumps of calli growing on callus cultures in a 3 x 3 cm area on the surface of a Baxter S/P filter and place on an osmotic induction media at 27°C in darkness.

11. Prepare 10 plates for each transgenic line, including the addition plate bombarded with plasmids encoding anthocyanin regulators pR and pC1.

Note: Score red spots 16-40 hours after bombardment.

12. Remove filters from media, place onto sterile opened Petri dishes and spread 10 µl of 36 µl of gold-DNA-ethanol solution onto the surface of microcarrier.

13. Accelerate in vacuum at 27 psi against a wire mesh screen. Shoot each plate once at 650 psi and a second time at 1100 psi in a previously soaked isopropanol disc.

    Note: If gold residue is not seen, bombard for a third time.

14. Transfer filters holding calli onto osmotic media, incubate for 16 hours in darkness at 27°C and transfer again onto callus maintenance media (N61-100-25) for 1-2 days to promote tissue recovery, prior to herbicide selection.

Herbicide Selection

1. Remove calli from the bombardment filters and place onto N6 2-0-0 containing 3 mg/ml bialaphos.

   Note: N6 2-0-0 contains N6 salts + vitamins, 2 mg/ml 2,4-D, and no proline or casamino acids.

2. Nonselectively subculture the tissues every 12 days for 10-12 weeks. After performing the first subculture, partially flatten on media to promote direct contact with herbicide.

3. After 6-8 weeks on herbicide white embryogenic sectors should be visible. Allow resistant sectors to grow to 1 cm diameter to proliferate enough to occupy the entire plate and subculture every 2 weeks on fresh herbicide media.

   Note: If necessary, add 0.4M sorbitol to media and incubate for 1-5 days to promote embryogenic sector induction.

4. Check individual resistant callus line by RNA gel blot hybridization for transgene expression.

Associated Products

- Spermidine Trihydrochloride (GoldBio Catalog # S-715)
- Bialaphos (GoldBio Catalog # B0178)
- D-Mannitol (GoldBio Catalog # M-060)

References

Fromm, M. E., Morrish, F., Armstrong, C., Williams, R., Thomas, J. & Klein, T. M. (1990). Inheritance and expression of chimeric genes in the progeny of transgenic maize plants. *Nature Biotechnology, 8*(9), 833-839. Doi:10.1038/nbt0990-833.

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