Protocol

TD-P Revision 3.0

Creation Date: 5/26/2016
Revision Date: 8/22/2018

Selection of Transformed Wheat Calli and Transgenic Plant Recovery

Adapted from Bohorova et al. (1999)

Introduction

Advances in biotechnology have paved the way for the development of techniques aiming to improve plants and their ability to withstand environmental stresses or epidemiological diseases that would otherwise lead to the destruction of species and the loss millions of plants. One of these techniques, biolistic transformation, is a widely used method for introduction of genes into plants that are resistant to *Agrobacterium*-mediated transformation. This technique promotes gene transfer through direct delivery into immature plant embryos or calli, leading to selection of transformed embryos and a final regeneration into complete plants. Here, we detail the creation of a selection medium for screening of transformed plants using the herbicides phosphinothricin or bialaphos.

Materials

- Plant cells (both transformed and nontransformed)
- Phosphinothricin (PPT) (GoldBio Catalog # P-165)
- Bialaphos (GoldBio Catalog # B0178)
- Media: MSE3, MSE5, MSR and MSE
- 10x MS macroelements (Macronutrients)
- 10x MS microelements (Micronutrients)

Preparation of MSE3 medium

- 100 ml 10x MS macroelements
- 100 ml 10x MS microelements
- 1 ml 1000x MS vitamins
- 0.040 mg Thiamine HCl (GoldBio Catalog # T-260)
- 150 mg L-Asparagine (GoldBio Catalog # A-357)
- 30 g Sucrose
- 2.5 ml 1mg/ml stock solution 2,4-Dichlorophenoxyacetic acid (2,4-D)
- 8 g Agar (GoldBio Catalog # P1001.0100)

> **Note: 2,4-D Note: 2,4-D can be toxic and should be handled with caution. Handle according to manufacturer's instructions.**

Preparation of MSE5 medium

- 4.43 g MS basal salt mixture

- 0.100 g Myo-inositol (GoldBio Catalog # I-525)
- 0.075 g Glycine (GoldBio Catalog # G-630)
- 0.877 g L-Glutamine
- 0.266 g L-Aspartic acid
- 0.228 g L-Arginine (GoldBio Catalog # A-030)
- 2 ml 1 mg/ml 2,4-D
- 0.2 ml Kinetin (GoldBio Catalog # K-100)
- 0.1 ml Gibberellic acid (GoldBio Catalog # G-120)
- 30 g Sucrose
- 8 g Agar

Preparation of MSR medium

- 4.43 g MS salts
- 0.040 mg Thiamine HCl
- 0.15 g L-Asparagine
- 20 g Sucrose
- 0.5 ml (1 mg/ml) Indole Acetic Acid (IAA) (GoldBio Catalog # I-110)
- 1 ml (1 mg/ml) 6-Benzylaminopurine (BAP) (GoldBio Catalog # B-110)
- 8 g agar

Preparation of MSE medium

- 50 ml 10x MS macroelements
- 100 ml 10x MS microelements
- 20 g Sucrose
- 1 ml IAA
- 8 g agar
- This solution can be stored at room temperature for 1 month.

Method

Preparation of MSE3 medium

1. Pour 500 ml deionized water into a 2 L beaker.

2. Add MS macroelements, MS microelements, MS vitamins, thiamine HCl, asparagine, and sucrose. Mix well.

3. Add 2,4-D (stock solution prepared by dissolving 10 mg 2,4-D into 1 ml NaOH and adding 9 ml deionized water).

4. Pour solution into a 1 L graduated cylinder and fill up to 1,000 ml deionized water.

5. Adjust pH to 5.7 with 1M NaOH.

6. Dispense 1,000 ml MSE3 medium into 2 1 L flasks (500 ml into each), and add 4 g agar into each and cap with foil.

7. Autoclave at 121°C for 15 minutes at 15 psi.

8. Cool the medium by placing the flasks in a 50°C water bath. Each flask containing 500 ml of medium is enough for 20 100 x 15 mm or 40 60 x 15 mm petri dishes. This medium can be stored at room temperature.

Preparation of MSE5 medium

1. Pour 500 ml deonized water into a 2 L beaker.

2. Add MS basal salts, myo-inositol, kinetin, gibberellic acid, 2,4-D and sucrose. Mix well.

3. Pour solution into 1 L graduated cylinder and fill to 900 ml with deionized water.

4. Adjust pH to 5.7 with 1N NaOH.

5. Pour 900 ml MS medium into two 1 L flasks, 450 ml each, and add 4 g agar into each cylinder.

6. Autoclave at 121°C for 15 minutes at 15 psi.

7. Cool the medium by placing the flasks in a 50°C water bath.

8. Pour 50 ml deionized water into a 200 ml beaker and add 0.075 g glycine, 0.877 g L-glutamine, 0.266 g L-aspartic acid (to dissolve completely add 0.266 g L-aspartic acid to

1 ml NaOH) and mix well. Pour this solution into a 100 ml cylinder and fill up to 100 ml with deionized water, using a 0.2 µm filter.

9. Add 50 ml from solution prepared in step 8 into each 450 ml cooled medium.

10. Mix well and pour into petri dishes. Each 500 ml of medium is enough for 20-100 x 15 mm or 40-60 x 15 mm petri dishes.

Preparation of MSR medium

1. Pour 300 ml deionized water into a 500 ml cylinder and add MS salts mixture, thiamine HCl, L-asparagine, sucrose, and IAA. Fill up to 500 ml with deionized water and mix until completely dissolved.

2. Adjust the pH to 5.7 with 1N NaOH.

3. Pour 500 ml deionized water into a 2 L beaker, add agar and melt in microwave.

4. Add solution prepared in step 1 to solution prepared in step 3 and mix well.

5. Pour the solution into sterilized baby food jars (30 ml/jar) or into Magenta vessels (40 ml/vessel), cover the containers with lids and autoclave for 15 minutes at 15 psi.

6. Allow the medium to cool and solidify in the jars/vessels.

Preparation of MSE medium

1. Pour 300 ml deionized water into a 1 L beaker and add macroelements from 10x MS basal salt macronutrient solution, MS microelements from 10x MS basal microelements solution, sucrose, and IAA.

2. Fill to 500 ml with deionized water and mix well.

3. Adjust the pH to 5.7 with 1M NaOH.

4. Place 8 g agar in a 1 L flask and add 500 ml deionized water. Melt in microwave.

5. Once melted, add the first solution to the melted agar and mix.

6. Pour this solution into baby food jars (30 ml/jar) or into Magenta vessels (40 ml/vessel), cover and autoclave for 15 minutes at 15 psi.

7. Allow the medium to cool and solidify at room temperature in the jars/vessels.

Selection

1. After bombardment, transfer immature embryos individually to the MSE3 medium and maintain for 7-14 days.

   Note: PPT inhibits glutamine synthesis causing a rapid accumulation of ammonia that leads to plant cell death. Bialaphos is a tripeptide antibiotic that consists of PPT and 2 L-alanine-residues. Bialaphos is an effective agent for selection of transgenic wheat calli, but does not always kill untransformed cells. To achieve optimum selection, subculture the bombarded cells at low density, ~10 immature embryos per dish.

2. Transfer embryos onto MSE5 medium containing 5 mg/L PPT or bialaphos for the first selection medium and culture for 2 weeks in darkness.

   Note: A range of concentrations from 1 to 10 µg/ml of PPT or bialaphos should be tested with nontransformed calli to get a more efficient selection system.

   Note: Approximately 20 target plates should be prepared for each experiment. Two plates are control, one grown on MSE5 to monitor the health of the culture and the second grown on the same medium containing 5-10 µg/ml bialaphos to demonstrate response of untransformed cells.

3. Discard calli that fail to grow.

4. Excise with scalpel cell clusters that have proliferated on MSE5 medium.

5. Subculture and transfer them onto MSE5 medium supplemented with 10 mg/L bialaphos or PPT. Maintain for 4 weeks.

   Note: Duration of the selection process is ~50-75 days on the respective medium with 5 and 10 mg/L PPT or bialaphos.

Plant Regeneration

1. Plant regeneration from resistant calli is performed on MSR medium, except that 5 µg/ml bialaphos is added to the medium.

2. The somatic embryos capable of developing into green shoots within 2-4 weeks are considered as putative transformants. Transfer these selected plantlets to MSE medium for root formation, but supplement with 1 µg/ml bialaphos to continue the selection.

3. Transfer the plantlets that develop on this medium to controlled growth chambers and greenhouse conditions to grow and for further analyses.

Associated Products

- Phosphinothricin (PPT) (GoldBio Catalog # P-165)
- Bialaphos (GoldBio Catalog # B0178)
- Thiamine HCl (GoldBio Catalog # T-260)
- L-Asparagine (GoldBio Catalog # A-357)
- Agar (GoldBio Catalog # P1001.0100)
- Myo-inositol (GoldBio Catalog # I-525)
- Glycine (GoldBio Catalog # G-630)
- L-Arginine (GoldBio Catalog # A-030)
- Kinetin (GoldBio Catalog # K-100)
- Gibberellic acid (GoldBio Catalog # G-120)
- Indole Acetic Acid (IAA) (GoldBio Catalog # I-110)
- 6-Benzylaminopurine (BAP) (GoldBio Catalog # B-110)

References

Bohorova, N., Fennell, S., McLean, S.D., Pellegrineschi, A., and Hoisington, D.A. (1999). Selection of Transformed Wheat Embryos/Calli and Recovery of Transgenic Plants. Laboratory Protocols: CIMMYT Applied Genetic Engineering Laboratory. International Maize and Wheat Improvement Center (CIMMYT), Mexico City, Mexico.

Murashige, T. and Skoog, F. (1962). A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiologia Plantarum,* 15(3), 473-497. Doi:10.1111/j.1399-3054.1962.tb08052.x.