Protocol

TD-P Revision 3.0

Creation Date: 3/30/2016
Revision Date: 8/10/2018

Agrobacterium tumefaciens-mediated transformation (AtMT) of *Colletotrichum graminicola*

Modified from Flowers and Vaillancourt (2005)

Introduction

Agrobacterium tumefaciens is a gram-negative bacteria and plant pathogen that typically causes tumor-like growths in infected plants. These tumors are prompted by the movement of transfer DNA (T-DNA) from the plasmid to the genome of the host eukaryote (often fungi). This horizontal gene transfer system has become a useful technique for the insertion of modified DNA into cells to create genetically modified plant lines, and is known as *Agrobacterium tumefaciens*-mediated transformation or *At*MT. In this protocol, we outline the steps to create an environment that allows for the transfer of a desired gene sequence from *Agrobacterium tumefaciens* to the genome of a eukaryotic host, the fungus *Colletotrichum graminicola*.

Materials

- Kanamycin (GoldBio Catalog # K-120 **ES**)
- Cefotaxime (GoldBio Catalog # C-104 **ES**)
- Carbenicillin (GoldBio Catalog # C-103 **ES**)
- Hygromycin B (GoldBio Catalog # H-270 **ES**)
- Thiamine HCl (GoldBio Catalog # T-260 **ES**)
- Acetosyringone
- LB agar
- *Agrobacterium tumefaciens* bacteria
- *Colletotrichum graminicola* fungal culture
- Minimal media broth
- Initiation media
- Sterile milli-Q water

Preparation of media recipes
Minimal Media (MM) Broth 50X Stock:

- Dissolve into 750 ml of sterile milli-Q water one at a time
- 102.5 g K_2HPO_4
- 72.5 g KH_2PO_4
- 25 g $MgSO_4$ (7 H_2O)
- 25 g $(NH_4)_2SO_4$
- 7.5 g NaCl

E S: EZ-Pak and Solution available

- 0.125 g $FeSO_4$ (7 H_2O)
- 20 ml $CaCl_2$ solution (5 g calcium chloride hexahydrate in 20 ml dH_2O)
- Fill to 1 L

Note: For a 1X solution of MM take 1 ml of the 50X Minimal Media stock and add 49 ml of sterile milli-Q water and 0.5 g sucrose (1%) and autoclave. After autoclaving, the media can remain at room temperature. Immediately prior to use, add 5 µl Thiamine HCl (1 g/ml stock solution) and 5 µl of Kanamycin (50 mg/ml stock solution).

For **Induction Media (IM)** per 50 ml (10 tubes at 5 ml each):

- 2 ml Minimal Media 50X Stock
- 2 ml 1M MES (pH 5.3) previously autoclaved
- 0.5 ml 1M Glucose previously autoclaved
- 0.25 ml 100% Glycerol previously autoclaved
- Adjust volume to 50 ml with sterile milli-Q water.
- Autoclave and cool. Store at room temperature until needed.
- Add 10 µl AS (100mM stock) and 5 µl Thiamine HCl (1 g/ml stock solution) to each tube immediately before use.

For **Solid Induction Media** per 100 ml:

- 2 ml Minimal Media 50X Stock
- 4 ml 1M MES (pH 5.3) (40mM final concentration)
- 0.5 ml 1M Glucose (5mM final concentration)
- 0.5 ml 100% Glycerol (0.5% final concentration)
- Adjust volume to 100 ml then add 1.5 g agar.
- Autoclave and cool in 50-55°C water bath.
- Add 200 µl AS stock solution and 100 µl Thiamine HCl stock solution before pouring plates.

Stocks for vitamins and antibiotics:
Add any of the antibiotics and vitamins to cooled media prior to pouring agar or inoculating broth.

Kanamycin Stock Solution Protocol – 50 mg/ml

- Add 50 µl of Kanamycin stock solution to 50 ml media (Final concentration; 50 µg/ml).

Cefotaxime Stock Solution Protocol – 100 mg/ml

- Add 200 µl Cefotaxime stock solution to 100 ml media (Final concentration; 200 µg/ml).

Carbenicillin Stock Solution Protocol – 100 mg/ml

- Add 250 µl of Carbenicillin stock solution to 100 ml media (Final concentration; 250 µg/ml).

Hygromycin B Stock Solution Protocol – 100 mg/ml

- Add 250 µl Hygromycin stock solution to 100 ml media (Final concentration; 250 µg/ml).

Thiamine HCl – 1 g/ml Stock:

- Dissolve 1 g per 1 ml of sterile milli-Q water, filter, sterilize and store at -20°C.
- Use 5 µl per 5 ml of minimal media (Final concentration of 50 µg/ml).
- Use 100 µl per 100 ml of solid induction media.

Acetosyringone (AS) – 100mM Stock (mw. 196.20 g/mol):

- Dissolve in 95% ethanol and adjust volume with water.
- Dissolve 0.3924 g in 12 ml 95% ethanol, then add 8 ml of sterile milli-Q water to equal 20 ml.
- Filter Sterilize and store at -20°C.
- Use 5 µl in liquid IM; use 200 µl in solid IM.

Method

Fungal cultures

1. Grow wild-type *Colletotrichum graminicola* on potato dextrose agar (PDA) for 10-15 days at 23°C in continuous light.

Note: Ensure that fungal cultures and spore suspensions are prepared under sterile conditions.

2. Prepare Falcate spore suspensions by adding 1 ml sterile milli-Q water to the culture and gently rub the surface with a sterile pestle.

3. Recover the suspensions from the plate and filter through several layers of sterile cheesecloth.

4. Wash 3 times in sterile milli-Q water and count the spore number with a haemacytometer.

5. Adjust the concentration of spores to 1 x 10^6 spores/ml. Spores are now ready for use in transformation procedure.

Transformation

For the transformation procedure described here, *Agrobacterium tumefaciens* strain AGL-1 containing the plasmid pBin-GFP-hph was used and will be denoted as *Agrobacterium*.

1. Streak out the *Agrobacterium* onto LB agar containing 50 µg/ml Kanamycin (prepared by adding 500 µl of a 50 mg/ml stock solution of Kanamycin in 500 ml of media).

2. Incubate upside down at 28°C for 48 hours.

3. Using a long sterile loop, transfer an actively growing colony of *Agrobacterium* to 5 ml of MM Broth augmented with 50 µg/ml Thiamine HCl (5 µl of 1 g/ml stock solution) and 50 µg/ml Kanamycin (5 µl of Kanamycin stock solution).

4. Incubate on an orbital shaker at 250 rpm at 28°C for 48 hours.

5. Transfer 200 µl of the MM Broth Culture to 5 ml of Initiation Media (IM) containing 200µM Acetosyringone (AS, 10 µl of 100mM stock solution) and 50 µg/ml Thiamine HCl (5 µl of 1 g/ml stock solution).

6. Incubate on an orbital shaker at 250 rpm at 29°C for ~6 hours until the OD 600 reading is at least 0.25.

7. Co-cultivate 100 µl of the Agro IM culture and 100 µl of fungal spores (harvested from an actively growing plate, pelleted, washed twice and concentration adjusted to 1 x 10^6 spores/ml as described in section Fungal cultures) in a 1.5 ml micro centrifuge tube.

8. Allow the tube to sit on the lab bench while you are placing small pieces of nitrocellulose membrane filters (Millipore 0.45 µm HA) on the solid IM media containing 200µM Acetosyringone (200 µl of a 100µM stock solution of AS per 100 ml) and 50 µg/ml Thiamine HCl (100 µl of 1 g/ml stock solution of Thiamine HCl per 100ml). This takes about 20-25 minutes.

9. Drop 200 of the co-cultivated Agro/spore solution on the nitrocellulose pieces. Spread the solution across the pieces with a sterile glass rod, sterile plastic spreader or sterile blue micropestle.

10. Incubate the plates either on the lab bench or in a typical growing area for the fungus

you are transforming. Incubate for 2-5 days.

Note: Control for this experiment is 5 pieces of nitrocellulose on augmented IM media inoculated with spore suspension only. Incubate as described above.

11. Use sterile forceps to transfer about 12 nitrocellulose pieces onto PDA augmented with:
    a. 200 µg/ml Cefotaxime (100 µl of stock solution 200 mg/ml) added to each 100 ml of media.
    b. 250 µg/ml Carbenicillin (100 µl of stock solution 250 mg/ml) added to each 100 ml of media.
    c. 250 µg/ml Hygromycin B (100 µl of Stock Solution in 100 ml of PDA) added to each 100 ml of media.

12. Incubate the PDA plates either at room temperature or in the required growing conditions for the fungus (usually 5-10 days).

13. Transfer transformed colonies to PDA augmented with 250 µg/ml Hygromycin B.

Associated Products

- Carbenicillin Disodium (GoldBio Catalog # C-103)
- Cefotaxime Sodium (GoldBio Catalog # C-104)
- Hygromycin B (GoldBio Catalog # H-270)
- Kanamycin Monosulfate (GoldBio Catalog # K-120)
- Thiamine HCl (GoldBio Catalog # T-260)

References

Flowers, J. L. and Vaillancourt, L. J. (2005). Parameters affecting the efficiency of Agrobacterium tumefaciens-mediated transformation of Colletotrichum graminicola. *Current genetics*, 48(6), 380-388.

Hooykaas, P. J. (1988). Agrobacterium tumefaciens Ti Plasmid-Derived Plant Vectors for Dicotyledonous and Monocotyledonous Plants. *Vectors,* 517-538. Doi:10.1016/b978-0-409-90042-2.50032-5.

Idnurm, A., Bailey, A. M., Cairns, T. C., Elliott, C. E., Foster, G. D., Ianiri, G., and Jeon, J. (2017). A silver bullet in a golden age of functional genomics: The impact of Agrobacterium-mediated

transformation of fungi. *Fungal Biology and Biotechnology, 4*(1). Doi:10.1186/s40694-017-0035-0.