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Preparation and Genetic Transformation of Parastagonospora nodorum Protoplasts

Forked from Preparation and Genetic Transformation of Parastagonospora nodorum Protoplasts

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ABSTRACT

An Abstract

PROTOCOL MATERIALS

(Glucanex) Lysing Enzymes from Trichoderma harzianum Merck MilliporeSigma (Sigma-Aldrich) Catalog #L1412-10G

Step 3

PROTOCOL integer ID:
9087

1 # Day 1 – Overnight culture

Prepare an overnight culture of *S. nodorum* by inoculation of 100 mL CzV8CS liquid medium (250 mL flask) with a concentrated spore suspension (~10^8 spores). Incubate culture at 22ºC with shaking (140 rpm). Start culture after lunch on Day 1 to ensure a good yield of mycelium.

Protocol

NAME
CZV8CS - liquid medium for fungal culture

CREATED BY
Oliver Mead

PREVIEW

1.1

2 Prepare CzV8Cs plates (at least 6 are required per transformation)

Protocol

NAME
CZV8- fungal protoplast agar

CREATED BY
Oliver Mead

PREVIEW

2.1

3 Prepare the following solutions:

Stock solutions (do not filter sterilise)

A. 50 mL 1.2 M $MgSO_4.7H_2O$ (14.8 g / 50mL)

B. 20 mL 1.2M Sorbitol (4.32 g / 20 mL)

C. 1 M Tris pH 7.5 [2]

D. 1 mL 1M $CaCl_2$ (0.147 g / mL) [3]

1. **Wash solution 600 mM $MgSO_4$**

50 mL (~50 mL required per prep)
Add 25 mL Soln A to 25 mL milliQ water. Filter sterilise into sterile 50 ml tube.

1. **Glucanex solution 1.2 M $MgSO_4$, 10 mM phosphate buffer pH 5.8**

25 mL per prep
Add 0.04g NaH_2PO_4 to 20 mL soln A and adjust pH to 5.8 using NaOH. Add the NaOH drop by drop. A precipitate will form after each drop but will go back into solution after a couple of minutes stirring. What until precipitate has dissolved before recording pH and re-adjusting if required. Once pH is correct, make up volume to 25 mL with remaining soln A. Do not filter sterilise at this point.

On the morning of the transformation, add 0.375 g glucanex to the solution and shake well to resuspend. The solution can now be filter sterilised.

1. **Protoplast overlay solution 600 mM Sorbitol, 10 Tris pH 7.5**

5 mL per prep
Add 2.5 mL of soln B to 2.45 mL milliQ water and 50 ul soln C. Filter sterilise.

1. **1 M Sorbitol-tris solution [2]** **1 M Sorbitol, 10 Tris pH 7.5**

5 mL per prep
Add 4.17 mL soln B to 780 ul milliQ water and 50 uL soln C. Filter sterilse.

1. **STC buffer [2,3] 1.2 M Sorbitol, 10 mM $CaCl_2$, 10 Tris pH 7.5**

10 mL (~10 mL required per prep)
Add 9.8 mL soln B to 100 uL soln C and 100 uL soln D. Filter sterilise

1. **60% PEG solution [2,3] 60% PEG-4000, 10 mM $CaCl_2$, 10 Tris pH 7.5**

(5 mL generally enough for 3 preps)
Add 6 g PEG 4000 (BDH only) to 5.3 mL milliQ water, 100 ul soln C and 100 uL soln D. Filter sterilise.

Note that 1.2 mL is required for each transformation and thus all 10 mL is not likely to be needed.

(Glucanex) Lysing Enzymes from Trichoderma harzianum **Sigma Aldrich Catalog #L1412-10G**

4 # Day 2 – Protoplast preparation

1. Harvest mycelium by centrifugation in sterile 50 mL tubes (4kg/5 min/4ºC) using a benchtop centrifuge with a swing-out rotor. During this time, add glucanex to Glucanex solution and also place a Petri dish in the 28^{o}C incubator.

5 1. Remove supernatant and wash mycelium with ~ 50 mL filter-sterile 600 mM $MgSO_4$. Centrifuge as above for 5 min. Filter sterilise glucanex solution.

6 1. Remove supernatant and resuspend mycelium in one 50 mL tube, with 25 mL filter-sterile glucanex solution.

7 1. Incubate for 2h without agitation at 28ºC in a sterile Petri dish. During this stage, melt the top agar and allow to cool in 50^{o}C waterbath (the correct temperature is important).

Protocol

NAME

CZV8CS- top agar for fungal protoplast transformation

CREATED BY

Oliver Mead

PREVIEW

7.1

8 1. Transfer protoplasts to a 50 mL sterile tube by gently pouring. DO NOT PIPETTE. Then very gently overlay with 5 mL 600 mM sorbitol, 10 mM Tris, pH 7.5. Centrifuge at 4,000 g for 15 min at 4 ºC (benchtop centrifuge).

9 1. Carefully remove protoplasts from the interface, using a 1 mL pipet, to a sterile 10 mL tube and add an equal volume of 1 M sorbitol, 10 mM Tris, pH 7.5. Mix gently and centrifuge at 1,500 g for 5 min at 4ºC (benchtop centrifuge).

10 1. Wash protoplasts with 3 mL STC buffer (1,500 g/5 min/4ºC) and resuspend in 0.5 mL STC buffer. Use inversion to resuspend protoplasts and place on ice.

11 1. Typically the protoplasts are counted (either 1:100 or 1:250 dilutions in STC) and then diluted to 5×10^8 / mL. 100 mL of these are then used for subsequent transformations.

12 # Protoplast transformation

1. Add prepared DNA (3 ug for PCR product or 7.5 ug for linearised construct in a volume up to 125 uL in STC) to 100 uL protoplasts in a 1.5 mL Eppendorf tube. Mix protoplasts with DNA very gently by inversion. Incubate at room temperature for 15 min. Also prepare a control where and equal volume of STC buffer (not containing DNA) is added.

During this stage, the pre-warmed top agar can be dispensed in 5 mL aliquots into sterile 10 mL tubes and kept at 50^{o}C. 4 tubes will be needed for each transformation and 2 for each control.

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CZV8CS- top agar for fungal protoplast transformation

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12.1

13 1. Add 200 ul 60% PEG solution to the protoplasts and mix by inversion. Add a further 200 ul 60% PEG solution and a final 800 ul 60% PEG solution with mixing by inversion and gentle agitation after each addition. Incubate at room temperature for 15-20 min.

14 1. Transfer approximately a quarter of the transformation mix (normally around 300-350 uL) to 10 mL pre-warmed (50^{o}C) CzV8-top agar, mix and on to a CzV8 plates (pre-warmed to RT). Repeat for remaining transformation mix (should have poured 4 plates in total). Repeat for 2 plates using the control (no DNA) transformation. Incubate plates in the dark (wrap plates in aluminium foil) at 22ºC.

15 # Antibiotic overlay (~40 h after transformation for hyg, 24 h for phleo)

1. Melt second bottle of CzV8-top agar and cool to 50ºC.

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CZV8CS- top agar for fungal protoplast transformation

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PREVIEW

15.1

16 1. Add the appropriate amount (see below) of antibiotic to 60 mL CzV8-top agar and pour 10 mL over each of the transformed protoplast plates. Pour 10 mL over one of the control protoplast plates and leave the other control plate without antibiotic overlay.

Antibiotic amount

Stock solutions

Phleomycin (Cayla) 250 mg/mL

Hygromycin (Astral) 100 mg/mL

Required amount for *S. nodorum* (total concentration for the whole plate)

Phleo 50 ug/mL

Hyg 200 ug/mL

Plate agar volume 10 mL
Protoplast top agar volume 10 mL
Antibiotic overlay volume 10 mL
Total 30 mL

Prepare antibiotic overlay agar at 3x concentration required for the whole plate.

3x Phleo = 250 mg/mL i.e. 0.6 ul/ml of overlay (0.6 x 60 = 36ul)
3x Hyg = 100 mg/mL i.e. 6 ul/ml of overlay (6 x 60 = 360ul)

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1. Wrap plates in foil and wait until mycelium appears on the surface of the transformed protoplast plates. Growth should appear on the control plate without overlay in a day or so and there should be no growth on the control plate with antibiotic overlay.

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1. Sub-culture colonies as they appear – between one and three weeks.