Transformation of *Phytophthora infestans*

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Welcome to the exciting world of *P. infestans* transformation! While the protocol is a challenging one, you should have good success if you carefully follow the protocol and treat *P. infestans* gently. In other worlds, use the best media (fresh), start with vigorous cultures, and don't let *P. infestans* spores or protoplasts go too long without media, etc.

A. Supplies you will need

Rye-sucrose agar
Rye-sucrose agar with selection drug
ALBA (lima bean) broth, clarified by centrifugation
Rye-sucrose broth, clarified by centrifugation, with 1 M mannitol
Sterile 400 ml beakers
Sterile 35-50 µm nylon mesh
Sterile miracloth (or more nylon mesh)
Sterile 1 liter glass erlenmeyer flasks
Bent forceps
50 ml plastic centrifuge tubes
16 ml polystyrene tubes (not polypropylene)
Fry protoplasting buffer (PFB)
Lipofectin (Thermo Life Technologies)
1 M mannitol-10 mM Tris pH 7.5
50% PEG 3350
1 M $CaCl_2$
1 M Tris pH 7.5
Bent glass rod
Cellulase, β-glucanase
Millipore syringe filters
20 ml syringe
Hemocytometers

B. Preparation of cultures for protoplasting

1. Inoculate plates with *P. infestans.* For the inoculum, harvest sporangia from 3-week old plates initiated from agar plugs, by adding sterile water to the plates and rubbing with a glass rod; typically 10^5 sporangia are obtained from a 100-mm plate. Check the resulting sporangia for contamination (especially bacteria) under a microscope, and count using a hemocytometer.

2. Spread about 10^4 sporangia per plate on ten to twenty 150 mm rye-sucrose agar plates.

Notes: By starting with a spore inoculum, you will be able to recover sporangia that will germinate more synchronously than sporangia from a plate inoculated with plugs.

However, the sporangia inoculum approach is more prone to contamination.

To avoid contamination, supplement the media with 50 µg/ml ampicillin, 50 µg/ml and 10 µg/ml benomyl or PCNB, or 4 ml per liter of nystatin suspension. Usually we use the latter as an antifungal, but treat the nystatin carefully, as it is heat labile. Only add it to media just before pouring the plates (or in the case of broth media just before use). Also, don't combine benomyl and nystatin, which seems to inhibit P. infestans growth.

Contamination is the bane of this protocol. Never use a plate if you have concerns about it being contaminated. Sometimes it is hard to see a yeast colony, for example, under the P. infestans hyphal mat (be on the look-out for irregularities in the mat, which might be a sign of contamination). Never handle the plates outside of the containment hood! Airborne spores can enter a closed petri dish very easily!

V8 media (unclarified, 1.5% agar) can also be used. However, some isolates grow poorly on V8 media, so this is not advised.

Fresh media is recommended for this step. Even plates that are 5 days old don't seem to support ideal growth of P. infestans.

2. Wait 9-14 days before harvesting. Pour about 15 ml of sterile, room temperature water on each 150-mm plate and rub using a flamed glass rod spreader. Try not to rub the mycelia off the plate. Decant the liquid into a 1 liter flask. Repeat for the remaining plates.

Notes: Some plates or isolates adhere poorly to the agar. If a small amount of hyphae comes off the plate, don't worry. We will end up filtering out hyphal fragments later on. However, if most of the mycelia come off the plate, press out the liquid from the mycelial mat with a forceps or pipette. Pipette off the water (which will contain sporangia). Then add more water, and squeeze out the spores again. This is not the preferred method!

If your fingers get wet during this stage, you need to improve your technique! Also, be sure that you include antibiotics at the next step. One yeast or bacterial cell will become millions by the time the transformation is over.

The process of harvesting the sporangia should not take more than about 15 minutes. If things are going slowly, add a little ALBA broth to the water in the flask, so the sporangia don't start to starve. Keep Phytophthora happy! Eventually you will end up with a 1:1 ratio of water and ALBA, so keep track of the volume of ALBA that you add.

3. Remove any chunks of mycelia or agar from the spore suspension by pouring through a 50 or 70 µm nylon mesh held in a ring holder, resting on top of a 400 ml beaker.

Note: The mesh and holder are obtained from Biodesign of New York, Inc. (914-454-6610). The asexual sporangia are 10 to 20 µm wide and pass easily through the mesh. Removing the larger fragments of mycelia at this step reduces the number of inviable protoplasts obtained in later steps.

4. Add an equal volume of ALBA (amended Lima bean) media. Add additional water and ALBA to a final concentration of 3 to 4 x 10^5 asexual sporangia per ml. Add 50 µg/ml ampicillin, 50 µg/ml vancomycin, and 4 ml per liter nystatin suspension (Sigma). Distribute among sterile 1 liter flasks (about 150 ml each; typically 4 flasks are used). Incubate, stationary, at 18°C.

Notes: Cultures are set up at a high surface to volume ratio since this gives the best growth. ALBA media is used since it seems to give the best protoplasts.

IT IS ESSENTIAL that the ALBA be totally clarified. Any chunks in the media will end up going into the final transformation tube and will bind DNA. The media is clarified by centrifugation before autoclaving, but sometimes an additional precipitate forms later on. Consequently, make up the ALBA at least 2 days before use, to allow time for any precipitate to form. You can decant off most of the supernatant; the remaining liquid can be separated from the precipitate by centrifugation in sterile 50 ml tubes.

Avoid using ALBA that is more than a week old. Also, store the ALBA in the dark at 4°C or room temperature. If stored cold, let it warm up before use.

C. Preparing protoplasts

1. Knowing when your cultures are ready for harvesting is an art, but typically they will be ready 24-36 hours after inoculation. The germlings should form a thin mat that, if swirled gently, will lift off the bottom of the glass flask in one large layer. If the germlings stick to the glass, they will be too old for optimal results.

2. Take a drop from the media and carefully check for contamination under the microscope. DO NOT proceed further if contaminants are seen. You will just end up wasting time and money.

3. If the cultures are OK, harvest the young germlings by pouring the culture through 50 µm nylon mesh, placed on top of a 400 ml beaker. Scrape off the tissue from the mesh with a bent forceps (flamed in ethanol) and place in a 50 ml plastic conical centrifuge tube. Note the volume, which is typically 5-10 ml.

4. The flow-through will contain ungerminated sporangia. Save these for a transformation on the next day! Place them back in the glass flasks, and incubate another 36 hr. Often this can be repeated twice, *i.e.* you end up doing protoplasting on three separate days.

5. While the germlings are still sitting in the 1:1 ALBA, prepare the protoplasting enzymes. For every ml of germlings, prepare about 3 ml of enzyme mixture. Do this by weighing out the dry enzyme mix, and dissolving it in "Fry protoplasting buffer" (mix by inversion several times until the powder is dissolved). Then sterile-filter it into a new, sterile 50 ml tube using a 10-20 ml syringe fitted with a syringe filter, and let it sit at room temperature.

<u>"Fry protoplasting buffer" (FPB)</u>

0.4M mannitol
20mM KCl
20mM MES, pH 5.7
10mM CaCl2
Add water to 40 ml

enzymes:
5 mg/ml (final concentration) cellulase (Sigma, from *Trichoderma reesi,* #C8546)
10 mg/ml "β-glucanase" (Vinoflow NCE, Extralyse, and others).

Note: Over the years we have used a variety of β-glucanases, starting with Novozyme234, which stopped being made many years ago, and then Glucanex ("Lysing Enzyme from Trichoderma harzianum" from Sigma). We have found that enzymes made for wine clarification such as Vinoflow FCE from Novozymes and Extralyse (Laffort) work just as well and are inexpensive. It should be noted that the different enzymes may work optimally in different buffers. For example, while Novozyme digested optimally in a high salt buffer, the others digest well in mannitol-based buffers such as one recommended to us by Bill Fry's lab at Cornell.

The Millipore brand filter seems to clog more slowly than other brands. A typical experiment usually has 20-30 ml of protoplasting mix; we usually use a 20 ml syringe with one filter, and reuse it 2 times if needed.

6. Rinse the hyphae in "Fry protoplasting buffer (FPB)." To do this, pour 40 ml FPB into the tube containing the germlings. Invert twice. Pour back through the nylon mesh to eliminate remaining ungerminated sporangia. Pick up the tissue with forceps and place in the tube with the enzyme solution. Mix up/break up the tissue by agitation with a 1 ml pipette.

7. Cover the tube with foil and shake gently at 40-50 rpm on a orbital platform. At intervals, check an aliquot under the microscope. Start harvesting the protoplasts when the reaction is about 90% done (some of the protoplasts are not totally round, *i.e.* some may be "sausages").

Notes: Typically protoplasting takes about 20 minutes; I never go longer than 45 minutes. I usually take a preliminary look at the protoplasts after 20 min, using a haemocytometer. Then at 30 minutes I decide whether to start harvesting the protoplasts or wait about 5 more minutes. The older mycelia digests poorly, so don't wait for 100% digestion; start when it is about 90% complete.

I also make a "preliminary" calculation of the total number of protoplasts at this stage. This helps to determine how to set up the different DNA treatments.

The goal from now on is to move as quickly as possible, to get the protoplasts back into media as soon as possible. Always try to make your protoplasts as happy as you can!

8. Near the end of protoplasting, take a preliminary count of the protoplasts using a hemocytometer. This value will tell you how many DNA treatments are possible.

9. Pass the protoplast mixture through sterile Miracloth (or 50 µm nylon mesh), to remove hyphal fragments and many of the empty sporangial cases. Rubbing the tip of a 1 ml pipette on the underside of the mesh will speed the flow-through.

Notes: I place the Miracloth in a plastic holder, and place it over a 400 ml beaker or a 100 mm petri plate. However, you can also shove the miracloth into the top of a 50 ml centrifuge tube, but this is not advised since it may "pop out."

To prepare the Miracloth, cut into ca. 4 inch squares. Boil in water to help release contaminants, including detergents which may lyse the protoplasts. Rinse in water, and then autoclave in foil packets.

10. Spin the flow-through in a swinging bucket rotor for 4 min at 700 x g at room temperature (1450 RPM in the tabletop Beckman centrifuge). Carefully pour off the supernatant (the pellet may be "soft). Use a pipette to remove any remaining drops of enzyme mix.

11. Between harvesting stage or during the following washes, based on the preliminary count of protoplasts, start setting up the DNA for transformation (you will be transforming DNA- liposome mixtures, which take time to form). Place up to 30 µg DNA in a polystyrene tube and add water to a final volume of 40 µl. Add 60 µl Lipofectin and incubate at room temperature for about 15 min. The mixture should become cloudy.

Notes: It will take about 15 minutes for the liposomes to optimally form; time is not critical, 10-20 minutes are OK.

Most batches of Lipofectin stimulate transformation and do not negatively affect transformation rates. Occasionally we have had a bad batch; this is something to be on the look- out for.

A typical transformation uses 30 µg DNA and 10^7 protoplasts, but can be scaled up.

12. Resuspend the pelleted protoplasts in 30 ml FPB. I do this by resuspending the pellet in a few mls of FPB by gentle agitation with a pipette or by hitting with your finger, and then adding the rest of the FPB. Be gentle! DO NOT vortex! You may GENTLY pipette up and down once; it is not critical to totally resuspend the protoplasts until the actual transformation (Section E). Respin. Pour off the supernatant.

13. Resuspend in 15 ml FPC plus 15 ml MT (1 M Mannitol, 10 mM Tris pH 7.5). Respin for 4 min. Pour off the supernatant.

11. Resuspend in 30 ml MT plus 10 mM $CaCl_2$. Remove an aliquot and place on a hemacytometer. Respin. Determine the yield while waiting for the centrifuge.

During these spins about 1/2 of the protoplasts may be lost. I believe that this represents vacuolated protoplasts that would not regenerate anyway. The count that you get may be inaccurate—but this is an important step in interpreting the transformation efficiency.

E. Transformation

1. Resuspend the protoplasts in MT plus 10 mM $CaCl_2$, at 1-2 x 10^7 protoplasts per 0.7 ml (this is the amount for one treatment). At this stage, do a very good job of resuspending the protoplasts, by first agitating the tube with a finger and then by pipetting up and down NO MORE THAN TWICE. Calculate the resuspension volume at 0.7 ml per transformation, plus 0.35 ml for a control.

2. Add the protoplasts (ca. 0.7 ml) to the DNA. Mix gently by rolling the tube (or gently pipette up and down once). Wait 4 min at room temperature.

3. Slowly add ca. 0.7 ml of 50% PEG-MW 3350 (Sigma) containing 25 mM $CaCl_2$ and 10 mM Tris 7.5. The volume equals the volume of protoplasts added to the tube. Roll the tube while adding the PEG, and then invert once. Wait 4 min at room temperature.

Notes: The idea is to slowly add the PEG; it should take about 30 seconds. If you have several tubes, you can first add 1/4 of the peg, invert; then add another 1/4, invert; then add the rest, invert.

Sometimes batches of PEG are bad. Bad batches are often yellowish or have a non-neutral pH. Check this whenever a new bottle of PEG is opened.

The PEG is prepared by autoclaving, and THEN adding Tris and $CaCl_2$ from 1 M stocks.

4. To the DNA-protoplast-PEG mixture (now about 1.5 ml), add 2 ml of clarified rye media plus 1 M mannitol at room temperature. Invert once. Walt 1 min. Add 4 ml of rye plus 1M mannitol. Invert once. Pour into a 50 ml conical tube containing 25 ml clarified rye plus 1M mannitol, 50 µg/ml ampicillin, 50 µg/ml vancomycin, and 4 ml/liter nystatin suspension. The final volume should be ca. 35 ml. Lay the tube on its side and incubate overnight (20 hour minimum, 36 hour maximum) at 18°C.

Notes: this procedure is for transformation with liposomes. If you were to not use liposomes, resuspend the protoplasts in MT with 25 mM $CaCl_2$. Also, raise the $CaCl_2$ concentration in the PEG solution to 25 mM.

The idea of adding the rye media slowly is to slowly decrease the PEG concentration. If you scaled up the transformation step (more than 2 x 107 protoplasts per tube), remember to scale this up too. If protoplast concentrations are too high, regeneration is inhibited.

E. Plating

1. The next day, invert the regeneration tubes to mix, and remove a sample to view under the haemocytometer. Calculate the percent of protoplasts that have regenerated. This should be based on the number of ORIGINAL protoplasts and not just those that are persisting in the culture after regeneration. Be careful not to count germinated sporangia.

Notes: This is an important step to evaluate the success of the procedure. Count at least the no-DNA control and two DNA treated samples. "Good" rates range from 5-15%.

At this stage, also check closely for contamination.

2. Spin the regenerated cultures at 1000 x *g*, 5 min (1800-2000 rpm in the Beckman table-top). Pour off the supernatant, leaving about 1.5 ml of liquid behind. Resuspend the tissue in this mixture by agitating with a 1-2 ml pipette. Aliquot about 0.25 ml each of about six to eight 100 mm plates of selection media (rye plus G418, hygromycin, or streptomycin, depending on the vector, but NO mannitol). Spread very gently with a glass rod.

Notes: G418 selection works the best for P. infestans, followed by hygromycin and streptomycin. The concentration of these drugs needs to be determined for each isolate. Typically 4 to 8 µg/ml G418, 25 to 40 µg/m hygromycin, or 50 µg/ml streptomycin are used.

Use FRESH media, no more than 24 hours old, for selection. While the standard media is rye media, pea media may also work. If you want to try pea media, here is the recipe:

- Autoclave 120 g frozen peas in about 600 ml distilled water
- Filter through 4 layers of miracloth. (solution can be frozen at -20C)
- Bring volume to 1 liter and add 2 g $CaCO_3$, 15 g agar, and autoclave.

3. Incubate plates at 18°C. Keep the plates right-side up until the liquid is absorbed, sometimes up to one day, and then invert the plates.

4. Colonies typically appear after 7 to 9 days. Transfer these to fresh plates with drug selection to confirm the transformed phenotype. Take care to carefully separate closely-spaced colonies to avoid cross-contamination. Colonies that arise after 2 weeks, which are often "wispy" and grow high and thin, are often not real transformants.