An improved protocol for the protoplast method of stable DNA transformation of *Phytophthora infestans*

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Protocol was adapted from Judelson et al. 1991. MPMI 4:602

Background

The first described method for stable transformation of *Phytophthora infestans* is based on introduction of DNA into protoplasts generated from germinated sporangia treated with the Novozyme 234 followed by germination of the protoplasts under antibiotic selection. This method became problematic since consistent batches of Novozyme 234 are no longer marketed. Nevertheless, the protoplast procedure remains critical for *P. infestans* research since it has more consistently generated homology dependent gene silencing than other methods, such as microprojectile bombardment, electroporation of zoospores, and *Agrobacterium*-mediated transformation. Here, we report a satisfactory alternative to Novozyme 234 and also new improvements of the general protoplast transformation protocol. This improved protocol has consistently produced transformants (up to 75 per experiment) and has been successful in generating strains with homology-dependent gene silencing and gene overexpression.

Citation

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Protocol

The protocol was adapted from Judelson et al. 1991. MPMI 4:602. Please consult the original protocol for more details.

The following steps should be performed under sterile conditions in biocontainment cabinet.

Isolation of young mycelia from 36-48 h germinated sporangia.

- Inoculate **60-65** rye/sucrose plates (big Petri dish) with 5 agar plugs each of the strain 88069, and grow at 18°C.
- After 14 day the mycelia should be ready to use for harvesting sporangia.
- Add sterile water (50 ml) to the plates and scrape off the sporangia with a rod made of glass. Remove this water and add to another plate. Do 4 plates at a time and then filter the sporangia through a nylon mesh of 60 µm to get rid of the hyphae. Collect the sporangia (which will pass through the filter) in a beaker in which a little 2X ALB media (Bruck et al., 1980) (aprox. 20 ml) has been poured.
- Adjust the concentration of sporangia to $2x10^5$/ml to $4x10^5$/ml of distilled water sterile.
- Add an equal amount of 2x ALB to the sporangia suspension. The final concentration should be $1X10^5$/ml to $2x10^5$/ml ALB (1X).
- Add antibiotics to the suspension: vancomycin 50µg/ml, ampicillin 50µg/ml, pimaricin 25µg/ml. Add not more than 200 ml of this suspension to a flask.
- Incubate at 18°C for 36-48 h.

Isolation of protoplasts and DNA treatment

Before starting, prepare the enzyme solution and PEG.

- Check mycelia for contamination. Collect by flask the mycelia on a 60 µm mesh. Sporangia that did not germinate the first day will be collected in a beaker and put back in the flasks. They can be used to do a second transformation the next day; generally **3-6** transformations can be performed with one bath of sporangia) and put in 1 tube sterile 15 ml (Falcon, orange cup) with a **10 ml** KC.
- Centrifuge 2 minutes 500 g 18°C.
- Remove the KC and mixed (very gently) in **10 ml** enzyme solution.
- Cover tube with aluminum foil and incubate **45-60** minutes at 40 rpm at room temperature.
- During incubation, prepare liposomes (**60 µl** Lipofectine® Gibco BRL® + **240 µl** H_2O sterile in **polystyrene (essential!) tubes**, don't pipet more), MT/KC and defrost transformation vectors.
- When the digestion is complete, filter the suspension through a 30 µm mesh to remove debris. Generally it is better to stop the digestion too early than too late. The protoplasts don't have to be completely round.
- Pellet the protoplasts in a swinging bucket rotor (1280 rpm) 3-5 minutes at 18°C. This will give a soft pellet (It is better to work as fast as possible, because than you get the highest efficiency of transformation).
- During the centrifugation, put **20-50µg** of transformation plasmid in Lipofectin®; let sit **around 30 minutes** at room temperature.
- Remove the supernatant and resuspend gently the pellet in **10 ml** of KC.
- Centrifuge 4 minutes at 1200 rpm.
- Remove the supernatant and resuspend gently the pellet in **10 ml** of (KC/ MT).
- Centrifuge 4 minutes at 1200 rpm.
- Remove the supernatant and resuspend gently the pellet in **10 ml** of MT.

It is absolutely not essential to get a complete dilution of the protoplasts before centrifugation a new time, in fact it is better to get the pellet loose from the bottom of the tube and just let it stay as a little lump in the tube.

- Centrifuge 4 minutes at 1280 rpm.
- After the last washing, Resuspend the protoplasts completely and ***very slowly*** in 1 ml of MT. (Always keep the protoplasts at room temperature).
- Add this mix to a polyester tube with liposomes and vector, roll nicely the tube and let sit for 5 minutes.
- Add 1 ml 50% PEG solution. Prepare fresh. Take the tube and turn it ***slowly around*** during the turning, add the PEG very slowly (this operation should last 30 seconds).
- Let the suspension sit and invert only once after 2 minutes and incubate 5 minutes.
- Add ***gently*** 2 ml of clarified Rye Sucrose media with 1 M Mannitol to the protoplasts. Invert ***gently*** one time to mix.
- After 2 minutes add ***gently*** 6 ml of clarified Rye Sucrose media with 1 M Mannitol, invert ***gently*** one time to mix.
- After 3 minutes distribute into a Petri dish containing 12 ml of the same media.
- Incubate **2-3** days at 18°C.

- Pellet the media/protoplast mixture 700g in the swinging rotator for 5 minutes. Poor off the liquid media and Resuspend (very gently) the regenerated protoplasts in 2 ml of clarified Rye Sucrose media with 1 M Mannitol.
- Spread the regenerated protoplasts onto rich Rye sucrose plates (5 big Petri dish) containing the selection antibiotic geneticin (G418) (for our strain 10 µg/ml) an antibiotic cocktail with vancomycin 50 µg/ml, ampicillin 50 µg/ml, pimaricin 25 µg/ml for be sure to not have contamination.

- After 9-20 days, transformed will appear. Pick the colonies for a secondary screen with a drug concentration 1.5-2 times higher than the selection concentration.

Solutions:

- Enzyme solution (in KC): - **5** mg/ml **Lysing Enzymes from *Trichoderma harzianum* (FLUKA)**
  - **2** mg/ml cellulase (Sigma®, de *Trichoderma reesei*)

  Solution sterilize with a 0.2 µm pore filter.
- Buffer KC: 0.64 M KCl – 0.2 M $CaCl_2$. Solution sterilize with autoclave.
- Buffer MT: 1 M mannitol – 10 mM Tris/HCl pH 7.5 – 20 mM $CaCl_2$. Solution sterilize with autoclave.
- PEG: 50% PEG 3350 (Sigma® Chemical Co. St. Louis, MO, USA) – 10 mM $CaCl_2$ – 10 mM Tris pH 7.5. Solution sterilize with a 0.2 µm pore filter.

ALB media:

K_2HPO_4	1.0 g	D+Glucose	5.0 g
KH_2PO_4	1.0 g	Vitamin stock	2.0 ml
KNO_3	3.0 g	Trace elements	2.0 ml
$MgSO_4$	0.5 g	Yeast extract	2.0 g
$CaCl_2$	0.1 g	Lima bean extract	250 ml
Sorbitol	5.0 g	Distilled H_2O qsp	1 liter
Mannitol	5.0 g		

Sterilize this solution by autoclave 20 min. 120°C, and after add under sterile conditions trace elements and vitamin stock. Stock at 4°C.

Lima bean extract:
Autoclave 150 g of frozen "baby" lima beans in 1 liter of distilled H_2O for 30 minutes. Strain through two layers of cheesecloth. Adjust at 1 liter, stock by 500 ml and autoclave again (Savage and al., 1968).Stock at 4°C. Keep frozen until needed.

Vitamin stock:

Biotin	0.20 mg
Folic acid	0.20 mg
l-inositol	12.0 mg
Nicotinic acid	60.0 mg
Pyridoxine-HCl	18.0 mg
Riboflavin	15.0 mg
Thiamine-HCl	38.0 mg
Coconut milk	50.0 ml
Distilled H_2O to make	300 ml

For start, filter coconut milk on miraclos and autoclave 20 min. 120°C. After, prepare vitamin solution in 150 ml of distilled water and filter under sterile conditions with Millex®-GP 0.22 µm (Millipore) in sterile bottle. Add 50.0 ml of coconut milk sterile (cold) and 100 ml of distilled H2O sterile.
Stock this solution at -20°C.

Trace elements:

$FeC_6H_5O_7.3H_2O$	215 mg
$ZnSO_4$.$7H_2O$	150 mg
$CuSO_4$	30 mg
$MnSO_4.H_2O$	15 mg
H_3BO_3	10 mg
MoO_3	7 mg
Distilled H_2O to make	400 ml

Sterilize this solution by autoclave 20 min. 120°C. Stock at RT°C.

$FeC_6H_5O_7.3H_2O$: Ferric citrate (Iron[III] citrate), Sigma F-3388

Preparation of Rye Sucrose Medium (RSM) 1M Mannitol

Day 1:

- For each litter of medium, sterilize 60 grams of rye in 3% bleach for 10 minutes and with agitation light.
- Rinse the rye in water and wash until the smell of bleach is gone (10 at 15 min).
- Place the seeds in a tray with water for 24 hours at RT°C.

Day 2:

- Grind the seeds add more water (≈ 800 ml final volume) and keep it at 68°C for 1 hour 15.
- Filter the seeds using 4 cheese cloths.
- Add 20 grams/liter sucrose.
- Use 1.5 l of RSM and aliquot equally over 2 centrifugation tubes (500 ml). Centrifuge 10 min 3500 rpm at room temperature.
- Weigh 182.17 g D(-)Mannitol Merck 5980.1000 and add clarified Rye Sucrose up to 1 l. Dissolve by stirring and heating. Divide in 4 bottles of 250 ml.
- Autoclave 20 minutes.

This solution is stock at 4°C.