Chapter 11

A Straightforward Protocol for Electro-transformation of *Phytophthora capsici* Zoospores

Edgar Huitema, Matthew Smoker, and Sophien Kamoun

Abstract

Genome sequencing combined with high-throughput functional analyses has proved vital in our quest to understand oomycete–plant interactions. With the identification of effector molecules from *Phytophthora* spp. we can now embark on dissecting the mechanisms by which effectors modulate host processes and thus ensure parasite fitness. One of the key limitations, however, is to genetically modify *Phytophthora* and assess gene function during parasitism. Here, we describe a straightforward protocol that allows rapid transformation of *Phytophthora capsici*, an emerging model in oomycete biology. *P. capsici* is a broad host range pathogen that can infect a wide variety of plants under lab conditions making it a suitable model for detailed studies on oomycete–host interactions. This protocol relies on electroporation-assisted uptake of DNA in to motile zoospores and allows the rapid identification and characterization of genetically stable transformants.

Key words: Oomycetes, Phytophthora capsici, Genetic transformation, Zoospore, Electroporation

1. Introduction

With the advent of genome sequencing and re-sequencing technologies as well as the availability of tools suited for high-throughput functional analyses, much progress has been made in understanding oomycete-plant interactions (1-3). It is now clear that all *Phytophthora* spp. examined to date, harbour vast arsenals of secreted proteins (effectors) that modulate host processes (4). One of the major objectives, therefore, is to determine the roles of these proteins and understand their function during infection. Strategies towards reaching these goals include (1) identification of host proteins that could be targeted and modified by each effector, (2) silencing or over-expression of effector genes to determine the impact on virulence, and (3) localization

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of *Phytophthora* (effector) proteins during infection. One key and rate-limiting step in performing these analyses is to genetically modify *Phytophthora*. Various protocols for DNA transformation of *Phytophthora* are available, but they are typically tedious, time consuming, and with variable reproducibility.

Genetic transformation of *Phytophthora* requires uptake of DNA and subsequent integration into the genome. DNA uptake takes place only in cells that lack intact cell walls and requires the formation of pores in the cell plasma membrane. Transformation of *Phytophthora* protoplasts using polyethylene glycol and CaCl₂ has historically been the method of choice (5). Here, we describe a rapid and efficient method towards the transformation of *Phytophthora capsici*. This method takes advantage of the fact that as part of its life cycle, *P. capsici*, similar to most other *Phytophthora* species, produces motile zoospores that lack cell walls and which are amenable to electroporation-assisted transformation.

1.1. Electroporation P. capsici transformation relies on the generation of large quantities of viable zoospores that lack cell walls. Before transformation, of P. capsici Zoospores *P. capsici* is grown on solid media plates under conditions that allow the formation of sporangia, structures that can be induced to release zoospores in aqueous suspensions. Sporangia are harvested by flooding plates with a cold wash solution, carefully dislodging sporangia and a gentle centrifugation and re-suspension step that concentrate sporangia. Sporangial solutions are then incubated at room temperature while exposed to an external light source, conditions that induce differentiation of sporangia and subsequent release of zoospores. Upon emergence of zoospores, suspensions are mixed with DNA and used directly for electroporation. Finally, electroporated suspensions are mixed with regeneration media and incubated overnight before being subjected to selection on plates.

Electroporation of zoospores using this protocol is feasible in other *Phytophthora* species in principle but is dependent upon the production of large number of zoospores, which does not occur with all strains. Optimal conditions for growth, sporulation, electroporation and subsequent selection should be determined first before embarking on transforming other *Phytophthora* species. The protocol described here can produce up to 40 *P. capsici* transformants per electroporation.

2. Materials

- 1. Unclarified 10% V8 agar: 100 ml V8 juice, 900 ml H_2O , 1 g of CaCO₃, 0.05 g β -sitosterol, 15 g agar/litre (see Note 1).
- 2. P. capsici strain LT1534 (see Note 2).

- 1× modified Petri's solution (6): 0.25 mM CaCl₂, 1 mM MgSO₄, 1 mM KH₂PO₄, 0.8 mM KCl/litre (see Note 3).
- 4. 15–20 µg of linearized plasmid DNA (see Note 4).
- 5. Gene pulser: BioRad Xcell & compatible Electroporation cuvettes, with gap width 4 mm (see Note 5).
- 6. Light microscope.
- 7. Haemocytometer slide.
- 8. Regeneration medium: 60 g organic rye grains, 20 g/l sucrose, 100 mM mannitol, 1 mM KCl, and 2.5 mM CaCl₂ (see Note 6).
- Selection medium: Unclarified 10% V8 agar with 50 μg/ml Carbenicillin, 25 μg/ml Vancomycin and 50 μg/ml G418 (geneticin) (see Note 7).

3. Methods

3.1. Culturing P. capsici	1. Grow <i>P. capsici</i> on large (15 cm) Petri dishes by placing 2–4 plugs of previously grown <i>Phytophthora</i> cultures onto the surface of non-selective unclarified V8 agar and seal with parafilm (see Note 8).
	 Incubate the plates in continuous light for 4 days at 25°C (see Note 9).
	 Remove the parafilm and incubate the cultures in the same conditions for at least 3 more days (see Note 9).
3.2. Preparation of Regeneration Media	1. Incubate 60 g of organically grown rye grains in 3% sodium hypochlorite for 10 min with light agitation (see Note 10).
	2. Decant and rinse the grains in running tap water for 10–15 min until the smell of bleach is completely absent (see Note 10).
	3. Cover the washed grains with distilled water (800 ml) and incubate at room temperature for 24 h.
	4. Transfer the suspension to a food blender and grind the grains for 1 min.
	5. Incubate the suspension for 2 h at 68°C.
	6. Centrifuge the suspension for 10 min at $3,300 \times g$ at room temperature, and collect supernatant.
	7. Filter supernatant through four layers of muslin and make volume up to 1 l (see Note 11).
	8. Add 20 g sucrose and autoclave.

- Upon use, take an aliquot of the required volume. (10 ml/ electroporation) and add: 100 mM mannitol, 1 mM KCl, and 2.5 mM CaCl₂ (see Note 6).
- *3.3. Electroporation of P. capsici Zoospores*
- 1. Flood a plate with 14 ml of ice-cold 1× modified Petri's solution and gently rub the mycelial mat with a sterile glass spreader to dislodge the sporangia.
 - 2. Tip the re-suspended sporangia solution onto the next plate and repeat step 1 until 3–4 plates have been harvested.
 - 3. Wash all the plates with an extra small volume of cold Petri's (5 ml) to harvest as many sporangia as possible. Between 5×10^5 and 1×10^6 /ml sporangia should be obtained. Collect all suspensions into a sterile 15 ml tube on ice.
 - 4. Divide the harvested sporangia solution into ice-cold 2-ml microcentrifuge tubes (one tube per electroporation) and centrifuge at $3,300 \times g$ for 5 min at room temperature.
 - 5. Remove excess supernatant to leave approximately 750 μ l and gently re-suspend the sporangial pellet.
 - 6. Place microcentrifuge tubes containing re-suspended sporangia onto a light box and incubate. Mix suspensions gently from time to time in order to keep sporangia in suspension.
 - 7. Evaluate the release and number of zoospores carefully every 5 min by placing a $10 \,\mu$ l droplet of suspension onto a haemocytometer and counting the number of swimming spores under a light microscope.
 - 8. Add DNA when zoospore numbers start to increase dramatically.
 - 9. As soon as there are 1×10^6 /ml zoospores, pipette the suspensions very carefully (see Notes 12 and 13) into pre-chilled 4 mm gap electroporation cuvettes.
- 10. Electroporate the suspensions with the following settings; Voltage, 550 V, capacitance, 50 μ F, resistance, 200 Ω . This should give a time constant between 4.8 and 6.0 ms.
- 11. Add 800 µl of ice cold, well-aerated regeneration medium, taking care to pipette slowly and carefully (see Note 14).
- 12. Transfer zoospore mix into 9 ml of ice-cold regeneration medium in a 15 ml centrifuge tube with lid. Lay the tube on its side and place onto a rocking platform at 18°C in the light and leave for 1 h to recover.
- 13. Add antibiotics: 25 μ g/ml vancomycin and 50 μ g/ml carbenicillin.
- 14. Place tubes back onto the rocking platform and incubate overnight.

- 15. Centrifuge 15 ml tubes for 5 min, $403 \times g$ at room temperature and remove the supernatant to leave between 3 and 4 ml per tube.
- 16. Disrupt the hyphal mats by pipetting vigorously. Start with a 1 ml pipette tip that has had its tip (approximately 3 mm from its end) cut off (using a hot sterile scalpel) and then with an intact 1 ml tip.
- 17. Plate the hyphal suspension onto selective V8 15 cm plates (at least 2 plates per electroporation) by dispensing the liquid across the plate. Limit mechanical manipulations on the plate surface as much as possible and allow excess liquid to air dry or soak into the plates (see Note 15).
- Seal the plates with parafilm and incubate the selective plates (inverted) at 25°C. Geneticin resistant colonies appear after 3–5 days and should be subcultured on fresh selective plates when possible.

4. Notes

- 1. Both calcium carbonate $(CaCO_3)$ and β -sitosterol do not completely dissolve in the media. Upon addition of all ingredients, as well as after sterilization, stir the media for 5–10 min. β -sitosterol is required for the production of viable zoospores. Omitting this component leads to the release of zoospore clumps that fail to separate.
- 2. *P. capsici* strain LT1534 is our preferred strain for transformation since this isolate grows vigorously on V8 and sporulates profusely using standard conditions. In addition, LT1534 was used in the *P. capsici* genome sequencing project.
- 3. It is recommended to make a sterile 50× concentrated solution of Petri's (12.5 mM CaCl₂, 50 mM MgSO₄, 50 mM KH₂PO₄, 40 mM KCl/litre) and dilute in sterile dH₂O prior to transformation. It is critical to use ice-cold Petri's solution for both harvesting and re-suspending sporangial suspensions.
- 4. We routinely use the pTOR vector for *P. capsici* transformation (7). pTOR constructs are maintained and isolated from *Escherichia coli* cells grown in Luria–Bertani (LB) media supplemented with Carbenicillin (100 μ g/ml). The pTOR cassette contains the NPTII gene for the selection of *Phytophthora* transformants on G418 (geneticin), a constitutive oomycete promoter (*ham34*) followed by a multiple cloning site to insert your gene(s) of interest and the *ham34* terminator. Although in other *Phytophthora* spp. linearized DNA is used

for transformation, we found that *P. capsici* transformation can be carried out successfully with undigested plasmid preparations.

- 5. We have successfully used the Genepulser Xcell system (Biorad) and compatible cuvettes for transformation experiments. It is recommended, however, to test other systems as we have successfully used the Micropulser for a limited number of experiments, and we have never performed side by side comparative experiments to find optimal transformation conditions.
- 6. For detailed instructions of regeneration media preparation, please refer to Subheading 3.1. It is imperative that mannitol, KCl, and CaCl, are added on the day of transformation.
- 7. For strain LT1534, we supplement V8 agar with 50 μ g/ml of G418 to select transformants. Before experiments are initiated with other *P. capsici* strains, we recommend establishing optimal G418 concentrations empirically. Carbenicillin and Vancomycin are added to limit the occurrence of bacterial contaminations during the zoospore preparation and electroporation process.
- 8. We use parafilm to prevent culture plates from drying.
- 9. We grow *P. capsici* at 25°C in LMS incubators fitted with interior illumination. Interior light intensity roughly corresponds to natural light conditions. Upon removal of parafilm and exposure to light, sporulation (formation of sporangio-phores bearing sporangia) is initiated. Sporulation is evident on plates when "powdery rings" are formed around the *Phytophthora* mycelial mat.
- 10. We strongly recommend using organically grown rye for the preparation of regeneration media. The use of pesticides on non-organically grown rye could impact the growth of *Phytophthora* and introduce variation between rye grain batches. In addition, after sterilization of grains, it is paramount that all traces of bleach are removed to obtain optimal regeneration and growth.
- 11. We commonly use 3–4 layers of cheesecloth to filter media supernatants. After centrifugation and filtration, media may still contain low levels of particulates that precipitate over time. This does not hamper regeneration.
- 12. Zoospores are very sensitive to physical stimuli. For example, excessive bouncing of zoospores onto solid surfaces results in immediate encystment. Therefore, care should always be taken when handling (e.g. mixing) zoospore suspensions.
- 13. Zoospore health is key for successful transformation. Actively swimming zoospores indicate healthy cultures, whereas rapid

(spontaneous) encystment is often observed under suboptimal conditions.

- 14. Aerate regeneration media before use to ensure optimal conditions for recovery. Once combined with electroporated zoospore suspensions, mix carefully to limit physical disruption of regenerating cysts.
- 15. We found that physically spreading young and regenerated mycelia onto solid surfaces (agar plates) resulted in reduced transformation efficiency. We therefore dry selection plates considerably before plating regenerated samples. We apply excess liquid onto the plates, spread the suspension by tilting the plates and allow the plates to soak up moisture.

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