Chapter 2

Electroporation and *Agrobacterium*-Mediated Spore Transformation

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Abstract

Genetic transformation is a key technology in modern fungal research. Most commonly, protoplasts are transformed using the polyethylene glycol-mediated transformation protocols. Because protoplasts are generated by treatment of mycelia with a crude enzyme preparation, the results tend to be inconsistent. Furthermore, some species cannot be transformed by this method. Electroporation (EP) and *Agrobacterium tumefaciens*-mediated transformation (AMT) are two alternative methods. These methods allow the transformation of spores or mycelia, they are simple to perform and provide consistent results. In this chapter, we describe EP and AMT protocols for the fungus *Colletotrichum gloeosporioides* f. sp. *aeschynomene* (*C. gloeosporioides*). These protocols can be used as baseline for the calibration of similar transformation protocols in other species.

Key words: Transformation, *Agrobacterium*, AMT, Electroporation, *Colletotrichum*

1. Introduction

Genetic transformation is an essential part of modern fungal research. Polyethylene glycol (PEG)-mediated protoplasts transformation has been the method of choice for many years and transformation protocols were developed in numerous species according to the original *Neurospora crassa* protocol (1). For a detailed protocol of protoplast transformation, see Chapter 1. However, although widely used, this system has several drawbacks. Primarily, protoplasts production and recovery might be problematic, especially in slow growing species. It is also well known that results vary considerably between different enzyme batches resulting in inconsistent transformation efficiencies. Alternative methods for direct transformation of spores or hyphae

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can provide efficient solution to these inherent problems. EP and AMT are the most commonly used alternatives. Both methods can be used to transform spores or hyphae, once working they are highly reproducible, and they have been used to transform a range of fungal species.

During EP, a high-voltage electric pulse creates a population of small, aqueous pores in the cell membrane through which DNA can enter the cell by diffusion or electrophoretically (2). The technique can be used to transform protoplasts as well as mycelia or spores (3–6). For spore transformation, the spores are usually pregerminated or incubated with a mild concentration of cell wall degrading enzymes (7–9).

Transformation of *Saccahromyces cerevisiae* by *Agrobacterium tumefaciens* was first reported in 1995 (10). Subsequently, it was demonstrated that AMT can be used to transform protoplasts as well as spores or mycelia of filamentous species (11). AMT is based on the natural ability of *A. tumefaciens* to transfect plant cells with a specific part of DNA (T-DNA). The discovery that *A. tumefaciens* can also transfect fungal cells attracted much interest in this method, and protocols were developed for numerous species, including member of Ascomycota, Basidiomycota, and Zygomycota, as well as Oomycetes (12). AMT solved transformation problems in a number of species such as *Agaricus bisporus* and *Sclerotinia sclerotiorum* and has become the method of choice when developing a new transformation protocol. AMT has also been reported to help solving problems of low homologous integration rates in certain species (13).

In this chapter, we describe an EP and AMT spore-transformation protocols. The protocols are specific to the fungus *C. gloeosporioides*, however, these protocols can be used as baseline for the calibration of similar procedures in various fungal species.

2. Materials

2.1. Agrobacterium-Mediated Transformation

2.1.1. Fungal Spores

1. Emerson's YpSs (EMS) agar medium. For 1,000 mL: yeast extract 4 g, soluble starch 2.5 g, $K_2HPO_4 \cdot 3H_2O$ 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, agar 16 g (see Note 1).

2.1.2. Agrobacterium

1. Antibiotics. Ampicillin (Amp), kanamycin (Kan), carbenicillin (Carb, Duchefa), and clafuran (Cla) are prepared in water as 100 mg/mL stock solutions and stored at −20°C. The final concentration for all antibiotics is 100 µg/mL (see Note 2).
2. Luria-Bertani (Miller) bacterial growth medium (LB) 25 g/L.

3. MES buffer. Prepare 1 M stock solution, pH 5.5: 625 g/L MES, titrate to pH 5.5 with 40% NaOH, store at 4°C under dark conditions.
4. Microelements solution. For 1,000 mL: 100 mg $ZnSO_4 \cdot 7H_2O$, 100 mg $MnSO_4 \cdot H_2O$, 100 mg $CuSO_4 \cdot 5H_2O$, 100 mg $Na_2MoO_4 \cdot 7H_2O$, 100 mg H_3BO_3. Store solution in a light protected bottle at 4°C.
5. 1% $CaCl_2$. Dissolve 0.3 g $CaCl_2$ in 30 mL water, autoclave and store at Room Temperature (RT).
6. 0.01% $FeSO_4$. Dissolve 0.02 g $FeSO_4$ in 200 mL water. Filter sterilize and store at RT in a light protected bottle.
7. 50% Glycerol. Dissolve 25 g in 40 mL water. Sterilize before use.
8. MN buffer. Dissolve 9 g $MgSO_4 \cdot 7H_2O$ and 4.5 g NaCl in 300 mL water and autoclave. Store at RT.
9. 20% NH_4NO_3. Dissolve 8 g of NH_4NO_3 in 40 mL water. Autoclave and store at RT.
10. 20% glucose. Dissolve 20 glucose in 100 mL water and autoclave. Store at RT.
11. Minimal medium (MM). For 800 mL (add in the following order): 0.64 mL K_2HPO_4 buffer 1.25 M, pH 4.8, 0.8 mL $CaCl_2$ 1%, 4 mL microelements solution, 8 mL $FeSO_4$ 0.01%, 32 mL MES buffer (1 M, pH 5.5), 8 mL glycerol 50%, 16 mL MN buffer, 2 mL NH_4NO_3 20% (w/v), 8 mL glucose 20% (w/v), water to 800 mL. Divide into 50 and 750 mL volumes. To the 50 mL volume, add 0.5 mL of 20% glucose and autoclave. To the 750 mL, add 11 g agar and autoclave.
12. Acetosyringone (Sigma). For 200 mM stock solution: dissolve 40 mg acetosyringone into 1 mL DMSO. Store in a light protected tube at –20°C. The final concentration is 200 μM.
13. Induction medium (IM): MM + 200 μM acetosyringone (see Note 3).
14. YENB. For 1,000 mL: 7.5 g yeast extract, 8 g nutrient broth, complete to 1 L with water.

2.1.3. Transformation of Agrobacterium

1. Plasmid DNA. A binary vector with T-DNA insertion sites should be used. Any commercial vector such as pBin19, pCAMBIA or equivalent can be used (see Note 4).
2. *Escherichia coli*. Propagate plasmid DNA in an *E. coli* commercial strain such as DH5α (Bethesda Research Laboratories) or equivalent.
3. Electroporator. All types of electroporators should be suitable. Use 0.2 cm cuvettes.

2.1.4. Fungal Transformation

1. Hemacytometer (Neubauer Improved Counting Chamber, 0.1 mm depth).
2. IM plates. To every 100 mL of MM agar (750 mL bottle, see Subheading 2.1.2, item 11) add 100 µL acetosyringone from stock solution. Keep plates in complete darkness.
3. Cellophane membranes. Use cellulose-based cellophane membranes (see Note 5). Cut 90 mm discs, place between two layers of wet filter paper and autoclave.
4. Regeneration medium (REG). For 1,000 mL: mannitol 145.7 g, yeast extract 4 g, soluble starch 1 g, agar 16 g, water to 1 L.

2.1.5. Selection of Transformants

1. Hygromycin B (Hyg). Prepare stock in water, 100 mg/mL (see Note 6).
2. REG + Hyg + Cla plates. To 100 mL of 50°C molten REG medium add 40 µL Hyg and 100 µL Cla. Mix thoroughly before plating. Keep plates in complete darkness.
3. EMS + Hyg. To 100 mL of 50°C molten EMS medium, add 100 µL Hyg. Mix thoroughly before plating. Keep plates in complete darkness.
4. Overlay agar + Cla. Prepare and autoclave 1% (w/v) water agar. To 100 mL of 50°C molten agar, add + 400 µL Cla.

2.2. Electroporation

All solutions must be autoclaved and stored at RT unless otherwise indicated.

2.2.1. Fungal Spores

1. Emerson's YpSs (EMS) agar medium. see Subheading 2.1.1, item 1.
2. Hemocytometer. see Subheading 2.1.4, item 1.
3. Pea extract (PE). Cook 800 g of frozen peas in a pressure cooker for 15 min, in 1.6 L water. Filter through Miracloth (Calbiochem), transfer liquid to bottles and autoclave for 30 min. Can be stored at RT for up to 1 month (see Note 7).

2.2.2. Fungal Transformation

1. Electroporation Buffer. For 1 L: HEPES 238 g, mannitol 9.108 g. Adjust pH to 7.5 with 1 M NaOH. Buffer must be cooled to 4°C before use.
2. Plasmid DNA. Prepare plasmid DNA in distilled water or electroporation buffer at a concentration of 1 µg/µL (see Note 8).
3. Electroporator. Small instruments such as the MicroPulser Electroporator (BioRad), which are used to transform bacteria are not suitable for most fungi. An instrument in which the voltage, resistance, and capacitance can be controlled, [e.g., Gene Pulser Xcell (BioRad)] should be used.

4. Liquid REG. See Subheading 2.1.4, item 4 without the agar.

2.2.3. Selection of Transformants

1. Hyg. see Subheading 2.1.5, item 1.
2. REG. see Subheading 2.1.4, item 4.
3. Overlay agar + Hyg. Prepare and autoclave 1% water agar. To 100 mL of 50°C molten agar, add 100 µL Hyg. Mix thoroughly before plating.
4. REG + Hyg plates. To 100 mL of 50°C molten REG medium, add 100 µL Hyg. Mix thoroughly before plating.

3. Methods

3.1. Agrobacterium-Mediated Transformation

3.1.1. Preparation of Fungal Spores

1. Grow the fungus on EMS plates for 5 days at 28°C (9) (see Note 9).
2. Wash plates with sterile distilled water. Transfer spore suspension to centrifuge tubes and spin at 6,000 × *g* for 10 min at 4°C.
3. Resuspend spores in 2 mL MM (control) or IM (MM + acetosyringone), count spores with the aid of a hemacytometer (dilute first if too dense) and bring to a final density of 10^8 spores/mL.
4. Dispense 100 µL of the spore suspension into Eppendorf tubes and place on ice.
5. Number tubes according to treatments.

3.1.2. Preparation of Competent Agrobacterium Cells

1. Plate *Agrobacterium* strain AGL1 from glycerol on LB agar plates + Kan + Carb. Incubate at 28°C for 2 days. For longer periods, keep plates at RT.
2. In the morning, inoculate 50 mL culture tubes containing 5 mL YENB + Kan + Carb with single colonies from plates. Grow cells at 28°C with agitation at 220 rpm.
3. In the evening, use the resulting starter to inoculate two 1 L flasks containing 200 mL of YENB + Kan + Carb. Grow cells at 28°C with agitation at 220 rpm overnight or until $O.D_{A595}$ 0.6–0.9.
4. Chill flasks on ice and transfer content to sterile centrifuge tubes.
5. Centrifuge at 4,000 × *g* for 10 min at 4°C, discard supernatant, combine the resulting pellets with the remaining supernatant and recentrifuge.
6. Resuspend cells in 50 mL of ice cold sterile distilled water, centrifuge at 4,000 × *g* for 10 min at 4°C, and discard the supernatant. Repeat this washing step one more time.

7. Resuspend pellet in 10 mL of 10% ice cold glycerol, centrifuge at 4,000 × *g* for 10 min at 4°C and discard the supernatant.
8. Resuspend cells in 2–3 mL of 10% ice cold glycerol, dispense 100 µL aliquots of the competent cells into Eppendorf tubes and store at –80°C.

3.1.3. Transformation of Agrobacterium with Plasmid DNA by Electroporation

1. Prepare sufficient amount of growth medium such as YENB (without antibiotics) for recovery of the electroporated cells. Adjust the electroporator settings to 2.5 kV, 25 µF, and 400 Ω, or follow the instructions of the electroporation apparatus if it has specific settings for *Agrobacterium*.
2. Chile cuvettes on ice, add 1 µg of plasmid DNA into 100 µL of competent cells and transfer the mixture to a cuvette (keep on ice). Dry the cuvette with a paper towel, place in the electroporation chamber and pulse once. When using an *Agrobacterium* specific program, simply follow the instructions.
3. Remove the cuvette from the chamber, immediately add 700 µL of sterile YENB medium and mix gently. Transfer the bacteria to an Eppendorf tube and incubate at 28°C for 3 h with agitation.
4. Plate 50–100 µL of cells on solid LB medium with the appropriate antibiotics to select for positive transformants. Incubate plates at 28°C for 2 days and then transfer colonies to separate plates.

3.1.4. PCR Analysis

Presence of the transforming vector in resulting colonies can be verified by "colony PCR." Prepare typical PCR reaction mix and aliquot 25 µL into each PCR tube (use each tube to analyse an individual colony). Place tubes on ice and transfer a small amount of colony to each tube using a toothpick or a 100 µL tip. After transferring of bacteria, leave tip inside the tube for 5–10 min (keep tubes on ice during the entire process). The amount of cells needed is small and sufficient mixing will result in complete cell lysis. Set the first cycle of PCR program conditions (single first cycle operating at 95°C) to 5–10 min to achieve initial cell breakage in addition to DNA denaturation.

3.1.5. Fungal Transfection

1. Pick a single colony from fresh plates and inoculate into 5 mL of LB + Kan + Carb. Culture overnight at 28°C with agitation at 220 rpm.
2. Centrifuge the culture, remove medium and resuspend in 2 mL LB *without antibiotics*. Divide the culture into two 50 mL tubes (2.5 mL each), dilute the bacteria to $OD_{A595} = 0.1$ with LB (control) or LB + 200 µM acetosyringone.

Incubate overnight under dark conditions with agitation at 220 rpm. Centrifuge the cells, remove the medium and dilute with MM (control) or IM to $OD_{A595} = 0.25–0.3$ (approximately 10^8 cells/mL).

3. Place sterile cellophane discs on MM plates (control) and on IM plates. Prepare four plates per each transformation treatment.
4. Mix 100 µL of bacteria suspension with 100 µL of spore suspension in an Eppendorf tube. Plate on the cellophane discs, 50 µL of the suspension per plate. Spread the suspension with glass beads or with a sterile dispensing stick. Incubate under dark conditions at 28°C for 48 h (see Note 10).
5. After the first incubation (48 h), transfer the cellophane discs onto REG without selection. Incubate plates for 18 h under light at 28°C (see Note 11).
6. Transfer the cellophane discs onto REG + Hyg (40 µg/mL) + Cla (100 µg/mL) (see Note 12). Incubate under dark conditions and watch for the appearance of colonies over 3–8 days (see Note 13).
7. When colonies appear on the plates, transfer a small piece (approximately 2 × 2 mm) onto fresh EMS + Hyg (100 µg/mL) + Cla (100 µg/mL) (see Note 14).
8. After 2 days, apply 1% agar + Cla (400 µg/mL) onto the plates. Incubate in light for 24 h, and then cut tips of hyphae that grow on the top agar layer and transfer to REG + Hyg (100 µg/mL) plates.
9. Check for insert using PCR or Southern blot according to conventional methods.

3.2. Electroporation

All steps in this protocol are carried under sterile conditions. It is also important to use large orifice tips in order to minimize damage to the conidia. Ampicillin (100 µg/mL) may be added to REG or EMS solid media to prevent bacterial contamination.

3.2.1. Preparation of Fungal Spores

1. Grow fresh fungal cultures on EMS plates for 5 days at 28°C under continuous fluorescent light. At this stage, the cultures sporulate profusely and spores germinate at high rates (see Note 15). After 5 days, harvest conidia by washing the plates with sterile distilled water.
2. Collect the liquid with spores from the plate, determine spore concentration with the aid of a hemacytometer (dilute first if too dense) and bring to a final density of 10^6 spores/mL in 50 mL of PE (see Note 16).
3. Dispense 50 mL spore suspension into 250 mL Erlenmeyer flasks and incubate for 2.5 h at 28°C with agitation at 190 rpm (see Note 17).

4. After 2.5 h, determine germination rate (see Note 18). Proceed only if germination rates exceed 50%.
5. Transfer the flasks to ice for 2 min to stop germination, and then transfer the cultures into 40 mL centrifuge tubes and centrifuge at 5,000 × *g* for 4 min at 4°C (see Note 19).
6. *Carefully* discard supernatant without disturbing the pellet. When using several tubes, combine all pellets (with small amount of supernatant) into a single tube, centrifuge again and discard the remaining supernatant.
7. Resuspend the pellet gently but thoroughly in 30 mL of ice cold sterile distilled water by pipetting (*do not vortex*) and centrifuge at 5,000 × *g* for 4 min at 4°C. Carefully discard the supernatant.
8. For each sample, prepare a 1.7 mL sterile Eppendorf tube and a 0.2 cm cuvette. Keep tubes and cuvettes on ice. Resuspend conidia by pipetting with sterile, ice cold Electroporation buffer (1 mL per sample).
9. Distribute 1 mL of the cell suspension into each tube (see Note 20). Centrifuge at 5,000 × *g* for 4 min at 4°C in a micro centrifuge, carefully remove the supernatant and then add 100 µL of sterile, ice cold Electroporation buffer to each tube.
10. Add 1 µg DNA to each tube and mix gently (see Note 21).
11. Incubate on ice for at least 10 min (can be extended to several hours if necessary). Always include negative (no DNA) and positive (a known plasmid) control treatments.

3.2.2. Transformation of Germinated Spores with Plasmid DNA

1. Prepare plates containing 20 mL of solid REG medium and a sterile, ice cold, REG with 5% PE.
2. Adjust the electroporator to the desired parameters; the optimal conditions for *C. gloeosporioides* 1.4 kV, 25 µF, 800 Ω (see Note 22).
3. Pipette the cells carefully inside the Eppendorf tube and transfer the mixture of cells and DNA to a cold, 0.2 cm electroporation cuvette.
4. Tap the spore suspension to the bottom of the cuvette, place the cuvette in the electroporation chamber and pulse once. Remove the cuvette from the chamber and immediately add 1 mL of sterile, ice cold REG with 5% PE medium.
5. Quickly but gently resuspend the cells, transfer back to the Eppendorf tube, and place on ice.
6. After 10 min, apply the content of each tube onto four plates of solid REG medium (250 µL per plate) and spread the cells using a sterile dispensing stick. Leave the plates in the sterile

hood until they are completely dry (about 30 min), and then incubate with lid facing down at 28°C (see Note 23).

7. After an overnight incubation, overlay with 10 mL of 1% agar with 100 µg/mL Hyg. Overlay half of the negative control plates with agar without the selection marker. Allow to dry and then incubate plates with lid facing down at 28°C. Colonies appear on top of the overlay after 4–6 days.

3.2.3. Analysis of Transformants

1. Transfer plugs of putative transformants to fresh selective medium (e.g., REG with 100 µg/mL Hyg) to verify if they are resistant to the drug. Always include wild type strain as control.
2. When using a reporter gene such as GFP, transformants can be identified by fluorescent microscopy. Otherwise, extract DNA from colonies and verify the presence of the transforming vector by PCR or Southern blot using conventional methods.

4. Notes

1. In other species, use appropriate sporulation medium.
2. Clafuran is a common drug and can be usually purchased from local pharmacies under the trade name Cefotaxime.
3. Prepare fresh before use.
4. For calibration of a new transformation protocol, it is recommended to use a plasmid with a GFP-expression cassette. This will allow easy and fast screening of practically unlimited number of colonies, to differentiate between transformants and background.
5. It is also possible to use nitrocellulose membranes; however, they are very expensive and have no advantage over cellophane. When using cellophane membranes, it is important to use only the type made up of natural cellulose which are used for backing and can be found in food stores. The synthetic cellophane (that is usually obtained in office supply stores) does not transfer the nutrients and should not be used.
6. This protocol describes only the use of the *HPH* (Hygromycin phosphotransferase) gene that confers resistance to hygromycin B as selectable marker. Other markers such as phleomycin or nourseothricin can be used in a similar way.
7. Pea extract (PE) can be prepared using either a pressure cooker or an autoclave for 15 min. Filtrate through Miracloth

and autoclave again for sterilization. Do not use immediately and allow to stand over-night for precipitation. It is advisable to use the medium for up to 1 month as well as avoiding its cooling due to crystallization and precipitation.

8. Linear or circular plasmid DNA can be used, although higher (up to 70%) transformation rates are obtained with linear DNA. Co-transformation with two or three plasmids is also possible.
9. When using other species, optimal conditions (medium, light, days) for the production of high-rate germinating spores must be determined.
10. Time of incubation may vary and should be determined experimentally. For most species 48 h should be fine, however fast growing species might be removed after 24 h to avoid overgrowth.
11. Normally, filters are transferred directly from the transformation plates (IM) to selection plates. In cases of weak growth on the selection plates and lack of colonies, it is recommended to add this extra step, which allows recovery of the fungus and expression of the resistance gene before exposure to the selective drug.
12. Hyg concentration is critical; too low concentration will result in high background and inability to distinguish transformants from the background, whereas at too high concentrations there will be no colonies. The optimal concentration should allow the development of low background after 24 h, but such that will not further develop after 48 h.
13. Colonies may appear already after 2 days, but should be transferred only when a clear colony has been produced, which usually takes at least 3 days. Additional colonies may appear during several days. After more than 8 days, colonies may develop that are not transgenic, and it is recommended to avoid such late developed colonies. The size of colonies may vary considerably, e.g., due to the number of colonies per plate. Ideally, there should be no more than ten colonies per 90 mm plate.
14. Colonies will appear as round, pink to dark color and should be clearly distinguished from background. When GFP is used, the colonies can be easily scanned using a fluorescent stereoscope and true transformants can be identified without opening the plate lids.
15. Achieving high rates of uniform germination is critical for this method. Therefore, the optimal stage for the production of high numbers of readily germinating spores must be determined.

16. Uniform germination is very important. If for any reason the spores are not germinated directly after harvest, they must be kept on ice until transferred to PE in order to prevent initiation of germination during preparations.
17. The amount of Erlenmeyers needed is half the amount of samples (cuvettes) used (e.g., for eight samples, four Erlenmeyers are needed). However, at least five Erlenmeyers should be used in order to pellet the cells properly.
18. In order to determine germination rates, 20 μL samples are taken out of the Erlenmeyers and viewed with light microscope. High germination rates are expected (up to 80% of conidia form aggregates and develop short germ tubes). If no or little germination is observed, incubation may be extended for an additional 20 min. Below 50% germination, the transformation will not work and it is recommended not to continue.
19. It is important to perform the following steps in sterile and ice cold conditions, thus utilization of sterile precooled solutions, cuvettes, and maintenance of spores on ice at all times are required.
20. The amount (mL) of electroporation buffer needed is the amount of samples (e.g., for ten samples, pelleted cells should be resuspended in 10 mL of buffer).
21. The highest number of transformants (up to 80 transformants/cuvette) is obtained with 1 μg DNA (9). When transforming with more than a single plasmid (co-transformation), use 1 μg of each plasmid. It is advisable to use as high concentration of the plasmid DNA as possible, and in any case the volume of plasmid DNA that is added to each cuvette should not exceed 4 μL in order to keep salt concentration low.
22. Transformation efficiency is affected by the rate of DNA uptake and cell viability. However, these parameters are in opposite correlation: DNA uptake increases with enhanced energy, whereas cell viability decreases. The optimal conditions for this protocol were determined after testing a wide range of conditions, in which each variable (voltage and resistance) was modified (see (9) for the complete set of conditions tested). It has been found that when parallel resistance was kept at 800 Ω, and voltage was varied between 1.25 and 1.75 kV, a primary peak in efficiency was observed at 1.4 kV.
23. Incubation period must be calibrated according to the selection marker. For Hyg, the optimal time is 12 h. Deviation from this time will result in either lack of growth (too short) or intense background (too long) and inability to isolate colonies.

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