

Sclerotinia sclerotiorum Protoplast Preparation and Transformation

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Abstract

Sclerotinia sclerotiorum causes white mold, leading to substantial losses on a wide variety of hosts around the world. Many genes encoding effector proteins play important roles in the pathogenesis of *S. sclerotiorum*. Therefore, establishment of a transformation system for the exploration of gene function is necessarily significant. Here, we introduce a modified protocol to acquire protoplasts for transformation and generate knockout strains, which complements the transformation system of *S. sclerotiorum*.

Keywords: *Sclerotinia sclerotiorum*, Protoplast, Preparation, Transformation, Gene function, Mutant

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Background

Sclerotinia sclerotiorum (Helotiales: Sclerotiniaceae), a phytopathogenic pathogen, infects more than 400 plants, including vegetables and ornamental plants (Kabbage et al., 2015). As the complete genome assembly of *S. sclerotiorum* is available, many genes encoding effector proteins have been preliminarily validated (Derbyshire et al., 2017). Preparation and transformation of protoplast are important measures to obtain mutants and research gene function. We introduce a protocol based on a standard protocol with minor modifications (Rollins, 2003). This method redounds to production of complete protoplasts for observation and transformation. Using this method, we successfully acquired *SsVPS51*, *SsDCTN1*, and *SsSAC1* knockout mutants, respectively (Qiao et al., 2021). Therefore, polyethylene glycol-mediated transformation is suitable for protoplast transformation of *S. sclerotiorum*, providing a convenient method for studying its genetics.

Materials and Reagents

1. 1.5 mL microfuge tube (Axygen)
2. 2.0 mL microfuge tube (Axygen)
3. Petri dish (9 cm diameter)
4. 40 µm cell strainer (Xiyang Co., Ltd., catalog number: 15-1040)
5. Syringe filter (Pall Corporation, catalog number: 4612-0.2 µm)
6. Sterile blade (ShanghaiLianHui Medical Instrument co., Ltd.)
7. 50 mL centrifuge tube
8. 50 mL conical flask
9. 100 mL conical flask
10. 250 mL conical flask
11. KCl (BIODEE, catalog number: DE-0395A-250 g)
12. CaCl₂ (BIODEE, catalog number: DE-0556A-500 g)
13. Agar A (BBI Life Science, catalog number: A600010-500 g)
14. Potato dextrose broth (Solarbio Life Sciences, catalog number: P9240)
15. D-sorbitol (Sigma-Aldrich, catalog number: S3889-500 g)
16. Tris-HCl (Sigma-Aldrich, catalog number: T6666-500 g)
17. Sucrose (BBI Life Science, catalog number: A100335-250 g)
18. Yeast extract (BBI Life Science, catalog number: A515245-500 g)
19. Hygromycin B (Thermo Fisher Scientific, Gibco™, catalog number: 10687010-20 mL)
20. Glucanex (Sigma-Aldrich, catalog number: L1412-5 g)
21. Polyethylene glycol 3500 (Sigma-Aldrich, catalog number: 25322-68-3-250 g)
22. Spermidine (Sigma-Aldrich, catalog number: 05292-1 mL)
23. Heparin (Sigma-Aldrich, catalog number: H3149-500 KU)
24. Casamino acid (Sangon Biotech, catalog number: A603060-100 g)
25. KCl buffer (pH = 6–6.5) (see Recipes)
26. 0.5% Glucanex (see Recipes)
27. Phanta Super-Fidelity DNA Polymerase (Vazyme, catalog number: P501-d2)
28. STC solution (see Recipes)
29. SPTC solution (see Recipes)
30. PDA medium (see Recipes)
31. PDB medium (see Recipes)
32. RM medium (see Recipes)
33. RMA medium (see Recipes)

Equipment

1. 1,000 μ L micropipette (Eppendorf)
2. 200 μ L micropipette (Eppendorf)
3. 10 μ L micropipette (Eppendorf)
4. Centrifuge (Eppendorf, model: 5424R)
5. Centrifuge (Eppendorf, model: 5810R)
6. Hemocytometer (Devan Scientific Co., Ltd., catalog number: AP-0650010)
7. Bacterial shaker incubator (Crystal Technology & Industries, model: IS-AX-190L)
8. Biochemical incubator (Shanghai Yuejin Medical Device Co., Ltd, model: HPX-B400)
9. Zeiss Axio Observer (Zeiss)

Procedure

A. Culturing and enrichment of *S. sclerotiorum* mycelium

1. Cut the PDA medium (see Recipes) where *S. sclerotiorum* grew on for three days into mycelium blocks (about 1 mm length $\times$ 1 mm width) (Figure 1).

*Note: The ideal cutting area is preferably at the edge of the fungal colony, where *S. sclerotiorum* hyphae actively grow.*

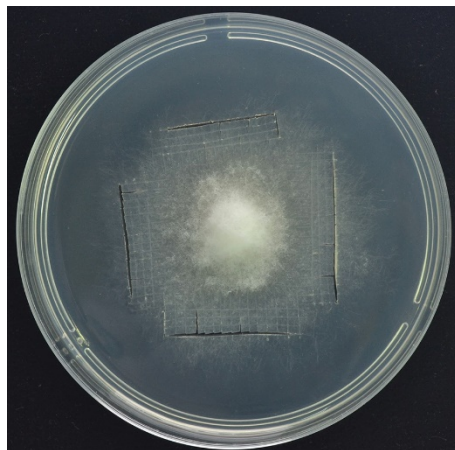


Figure 1. The ideal cutting area

2. Collect the mycelium blocks into a 250 mL conical flask containing 100 mL of PDB medium, and shake the flask to mix them together.
*Note: It's better to add approximately 200 mycelium blocks to 100 mL of PDB medium. This proportion guarantees that *S. sclerotiorum* uptakes adequate nutrition and produces enough fresh mycelium.*
3. Fasten the conical flask on a shaking table (180 $\times$ g, 25 $^{\circ}$ C) and culture for 14–20 h, until fresh mycelium grows from the mycelium blocks (Figure 2).

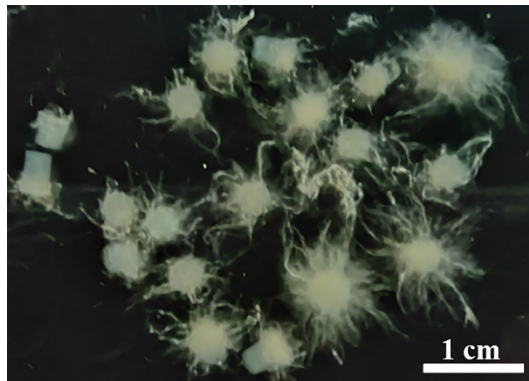


Figure 2. Fresh mycelium after culturing for 14–20 h. Scale bar = 1 cm.

4. Using a tweezer, separate fresh mycelium from mycelium blocks, and collect them in a 50 mL centrifuge tube.
5. Resuspend the mycelium with 20 mL of KCl buffer, centrifuge at $1,500 \times g$ and 25°C for 5 min, and then discard the supernatant.
6. Repeat step A5 once again.

B. Protoplast preparation

1. Add 20 mL of 0.5% Glucanex [lysing enzyme from *Trichoderma harzianum* (Sigma)] to a 50 mL centrifuge tube containing 1 g of mycelium material. Incubate with gentle shaking ($120 \times g$ on the rotator) in the dark at room temperature for 2–3 h.
2. After the enzymatic digestion, filter the digestion mixture using a cell strainer, and collect the filtrate into a 50 mL centrifuge tube (Figure 3A).

Note: Place the filtrate on ice and proceed cautiously and gently with the next steps, as protoplasts are fragile and may easily rupture (Figure 3B).

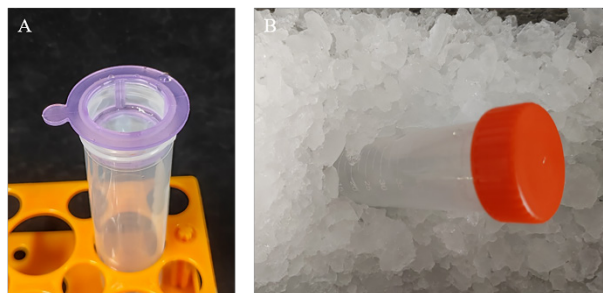


Figure 3. Filter the protoplasts and place the filtrate on ice

3. Centrifuge the filtrate at $1,200 \times g$ and 4°C for 10 min, and carefully discard the supernatant.
4. Wash the protoplasts gently with 5 mL of precooled KCl buffer, centrifuge at $5,000 \times g$ and 4°C for 2 min, and then discard the supernatant carefully.
5. Repeat step B4 once again.
Note: Steps B4 and B5 effectively remove the residual enzyme mixture.
6. Resuspend the sediment with 1 mL of precooled STC, centrifuge at $5,000 \times g$ and 4°C for 2 min, and discard the supernatant by slowly decanting the tube.
7. Resuspend the sediment gently with 800 μL of precooled STC buffer and add 200 μL of precooled SPTC buffer drop by drop with a pipette.

8. Place the protoplast solution on ice and check the status of the protoplasts with a Zeiss Axio Observer (Figure 4).

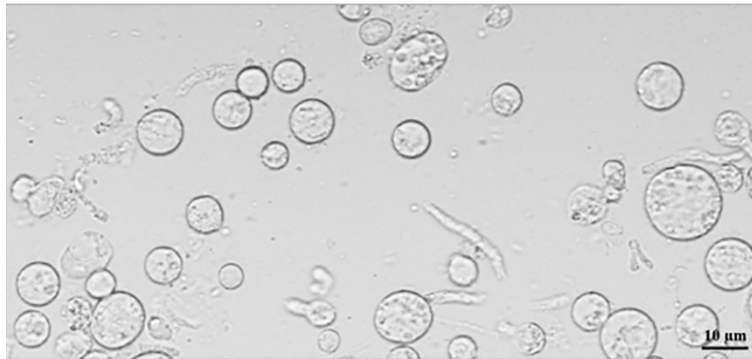


Figure 4. Protoplasts of *S. sclerotiorum*. Scale bar = 10 μ m.

C. Protoplast transformation

1. Resuspend the protoplasts to a concentration of 1×10^8 protoplast per milliliter, in four parts of STC and one part of SPTC.
Note: Calculate protoplast concentration with a hemocytometer, according to the manufacturer's instructions.
2. Add 5 μ g of transforming DNA, 5 μ L of spermidine (50 mM stock), and 5 μ L of heparin (5 mg/mL) to 100 μ L of protoplast suspension in a 1.5 mL microfuge tube, and then mix gently.
Note: Mix them together by flicking the bottom of the microfuge tube instead of using a pipette.
3. Incubate the mixture on ice for 30 min, then gently add 1 mL of SPTC solution, and incubate at room temperature for 20 min.
4. Transfer the transformation mixture into a 50 mL centrifuge tube, and add 10–20 mL of Regeneration Medium (RM).
5. Culture the mixture with $100 \times g$ agitation, at 25 $^{\circ}$ C overnight.
6. Gently mix the protoplasts with 200 mL of RMA medium at 43 $^{\circ}$ C, pour this into Petri dishes (10 mL per dish), and incubate at 25 $^{\circ}$ C for 24 h.
Note: Heat up RMA medium until it melts completely, and cool it down in a water bath set to 43 $^{\circ}$ C.
7. After, cover the plates with 10 mL of RMA medium containing 100 μ g/mL hygromycin B.
8. Culture at 25 $^{\circ}$ C for 7–10 days, and transfer the transformants to fresh PDA medium containing 100 μ g/mL hygromycin B.
9. Transfer the hyphal tip of hygromycin-resistant transformants to PDA medium containing 100 μ g/mL hygromycin B. Repeat the selection at least three times (Figure 5A).
Note: Pick the edge hyphae of recovered hygromycin-resistant transformants using a sterile blade for the first selection, and cut the mycelium block for the next two selections.

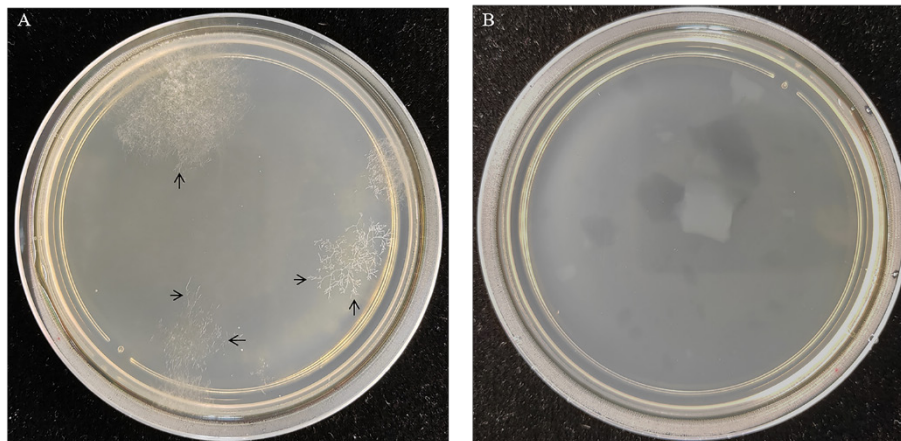


Figure 5. Colony morphology of *S. sclerotiorum* grown on selective RMA medium. A. Colony morphology denoting successful transformation. The hyphal tips labeled by black arrows can be transferred to PDA medium for selection. B. Negative control plate made by adding no DNA in step C2.

10. Extract total RNA of any colony and reverse transcript into cDNA. Perform PCR to validate positive colony by Phanta Super-Fidelity DNA Polymerase with the following cycles:
 - a. Pre-denature the cDNA at 95 °C for 30 s
 - b. Denature the cDNA at 95 °C for 30 s
 - c. 50 °C for 15 s
 - d. 72 °C for 90 s
 - e. Repeat steps b-d for 25–35 cycles
 - f. 72 °C for 10 min

Note: Primers designed to check mutants are as follows:

SsVPS51-GT-5'-F: CTTAGAGACTTGAGGAAGTC

SsVPS51-GT-3'-R: CTACTTCTATTGCTATTC

Recipes

1. KCl buffer (pH =6–6.5, sterilize by syringe filter, and store at 25 °C)

Reagent	Final concentration	Amount
KCl	0.6 M	4.47 g
CaCl ₂	50 mM	0.56 g
ddH ₂ O	n/a	up to 100 mL
Total	n/a	100 mL

2. 0.5% Glucanex (store at 4 °C)

Reagent	Final concentration	Amount
Glucanex	n/a	0.5 g
KCl buffer	n/a	up to 100 mL
Total	n/a	100 mL

3. STC solution (autoclave at 121 °C for 20 min)

Reagent	Final concentration	Amount
Sorbitol	0.8 M	145.74 g
Tris-HCl (pH = 8.0)	50 mM	7.88 g
CaCl ₂	50 mM	5.54 g
ddH ₂ O	n/a	up to 1,000 mL
Total	n/a	1,000 mL

4. SPTC solution (autoclave at 121 °C for 20 min)

Reagent	Final concentration	Amount
Sorbitol	0.8 M	145.74 g
Tris-HCl (pH = 8.0)	50 mM	7.88 g
CaCl ₂	50 mM	5.54 g
Polyethylene glycol 3500	0.11 M	400 g
ddH ₂ O	n/a	up to 1,000 mL
Total	n/a	1,000 mL

5. PDA medium (autoclave at 121 °C for 20 min)

Reagent	Final concentration	Amount
Potato dextrose broth	n/a	24 g
Agar A	n/a	15 g
ddH ₂ O	n/a	up to 1,000 mL
Total	n/a	1,000 mL

6. PDB medium (autoclave at 121 °C for 20 min)

Reagent	Final concentration	Amount
Potato dextrose broth	n/a	24 g
ddH ₂ O	n/a	up to 1,000 mL
Total	n/a	1,000 mL

7. RM medium (autoclave at 121 °C for 20 min)

Reagent	Final concentration	Amount
Sucrose	n/a	274 g
Yeast extract	n/a	1 g
Casamino acid	n/a	1 g
ddH ₂ O	n/a	up to 1,000 mL
Total	n/a	1,000 mL

8. RMA medium (autoclave at 121 °C for 20 min)

Reagent	Final concentration	Amount
Sucrose	n/a	274 g
Yeast extract	n/a	1 g
Casamino acid	n/a	1 g
Agar A	n/a	15 g
ddH ₂ O	n/a	up to 1,000 mL
Total	n/a	1,000 mL

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Competing interests

No financial, personal, or professional interests have influenced the work.

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