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Fusarium graminearum Double (Triple) Mutants Generation Using Sexual Crosses

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[Abstract] *Fusarium graminearum* is a destructive phytopathogen that infects major cereal crops such as wheat, maize and barley. Double or triple mutants are often very useful in the phenotypic and genetic analysis of genes that function redundantly or within similar pathways. When single gene mutants are available, double or triple mutants can be generated by crossing heterothallic strains or multiple rounds of protoplast transformation. When individual mutants carry different antibiotic resistance, it is convenient to use the sexual crossing to generate desired recombinant strains. Here, we present a protocol for generating double or triple mutants by sexual crossing in one homothallic strain with further antibiotic resistance and genomic DNA PCR screening of recombinant progenies.

Keywords: *Fusarium graminearum*, Sexual crossing, Double mutants, Triple mutants

[Background] The ascomycete fungus *Fusarium graminearum* is a devastating phytopathogen that causes head blight, ear rot, stalk rot and crown in cereals. It can produce perithecia on carrot agar *in vitro.* This assay can be used for studying perithecia development, ascospore discharge and sexual recombination (Nicholson, 2007).

F. graminearum is homothallic and has both MAT1-1 and MAT1-2-1 locus; each of these locus deletion mutants is sterile in self-crosses (Zheng *et al*., 2013). To generate double gene mutants, one single gene mutant has traditionally been outcrossed with *MAT* deletion mutants and further outcrossed with another single mutant (Bowden and Leslie, 1999; Lee *et al*., 2011; Son *et al*., 2012). Another strategy of double or triple mutants construct is deleting a gene in the other mutant strain with protoplast transformation (Oide *et al*., 2007 and 2014).

Here, we adapt and simplify this method, and present the details of the protocol to generate double and triple mutants using sexual crosses in one homothallic strain with further antibiotic resistance screening.

Materials and Reagents

1. 60 mm x 15 mm diameter plates (Sigma-Aldrich, catalog number: P5481-500EA)
   Manufacturer: Excel Scientific, catalog number: D-901.
2. Sterile glass spreading rod (CHEMGLASS, catalog number: CLS-1350-01)

3. 1.5 ml sterile centrifuge tube (Corning, Axygen®, catalog number: MCT-150-C)
4. Sterile toothpicks (Purchased from Carrefour Supermarket)
5. 90 mm diameter plates (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 263991)
6. Fungal strain: *F. graminearum* PH-1 (NRRL 31084)
7. Sterile ddH_2O
8. Fresh carrot (Purchased from Carrefour Supermarket)
9. TWEEN 60 (Sigma-Aldrich, catalog number: P1629)
10. Ampicillin sodium Salt (YEASEN, catalog number: 60203ES60)
11. Yeast extract (Oxoid, catalog number: LP0021)
12. Casamino acids (Sigma-Aldrich, catalog number: 22090)
13. Sucrose (Sinopharm Chemical Reagent, catalog number: 10021418)
14. Hygromycin B (Sigma-Aldrich, Roche Diagnostics, catalog number: 10843555001)
15. G418 sulfate (Santa Cruz Biotechnology, catalog number: sc-29065)
16. V8 juice (Campbell Soup, 051000000675)
17. $CaCO_3$ (Sigma-Aldrich, catalog number: C4830)
18. Nourseothricin sulfate (Goldbio, catalog number: N-500-100)
19. Fresh carrot agar (see Recipes, store at 4 °C)
20. 2.5% TWEEN 60 (see Recipes, store at RT)
21. TB3 plate (see Recipes, store at 4 °C)
22. V8 juice agar (see Recipes, store at 4 °C)

Equipment

1. 1 L Erlenmeyer Flask (Fisher Scientific, catalog number: S63274)
   Manufacturer: Corning, Pyrex®, model: 49801L/EMD.
2. 500 ml Erlenmeyer Flask (Fisher Scientific, catalog number: S63273)
   Manufacturer: Corning, Pyrex®, model: 4980500/EMD.
3. Hemocytometer (0.10 mm, 1/400 mm^2) (QIUJING, model: XB-K-25)
4. Biological safety cabinet (ESCO Micro, model: FHC1200A)
5. Autoclave (Zealway Instrument, model: GI54DWS)
6. Homogenizer (Ronghua Instrument Manufacturing, model: JJ-2B)
7. UV light (Philips Lighting, model: TL-D 15W/BLB 1SL/25, catalog number: 928024810803)
8. Induction chamber (JIANGNAN INSTRUMENT, model: GXZ-300)
9. Vortexer (Bio-Rad Laboratories, model: BR-2000 Vortexer)
10. Mold incubator (Yiheng, model: MJ-150I)
11. Microscope (Olympus, model: BX51)

Procedure

To generate triple mutants, we screened for three antibiotic resistance genes as selective markers: hygromycin B phosphotransferase (*hph*) gene conferring resistance to the antibiotic hygromycin, neomycin phosphotransferase (*npt*) gene for G418, and nourseothricin acetyltransferase (*nat*) gene for nourseothricin.

A. Generation of double mutants

1. Pick some mycelia from stock culture and inoculate carrot agar with two individual gene deletion mutants (A mutant with *hph,* B mutant with *npt*) on the opposite side of a 60 mm diameter plate (Figure 1A). Place the inoculated plate in a mold incubator at 25 °C in the dark.
2. Three days after inoculation, two colonies mycelia should reach each other (Figure 1B). Distribute 600 µl 2.5% TWEEN 60 to the surface of aerial mycelium (Figure 1C), press the aerial mycelium down with a sterile glass rod until the surface of the plate is smooth and the aerial mycelium is not visible anymore (Figure 1D).
3. Put the plate under UV light (12 h on/12 h off) in a chamber at 24 °C to induce perithecia formation (Figure 1E).
4. Check the plate every day after induction. If any aerial mycelia appear again, add 50-100 µl 2.5% TWEEN 60 and press down using glass rod.
5. Four days after induction, initial perithecia should be visible on the surface of the plate. The perithecia with mature ascospores usually form on the 7th day after induction (Figure 1F).

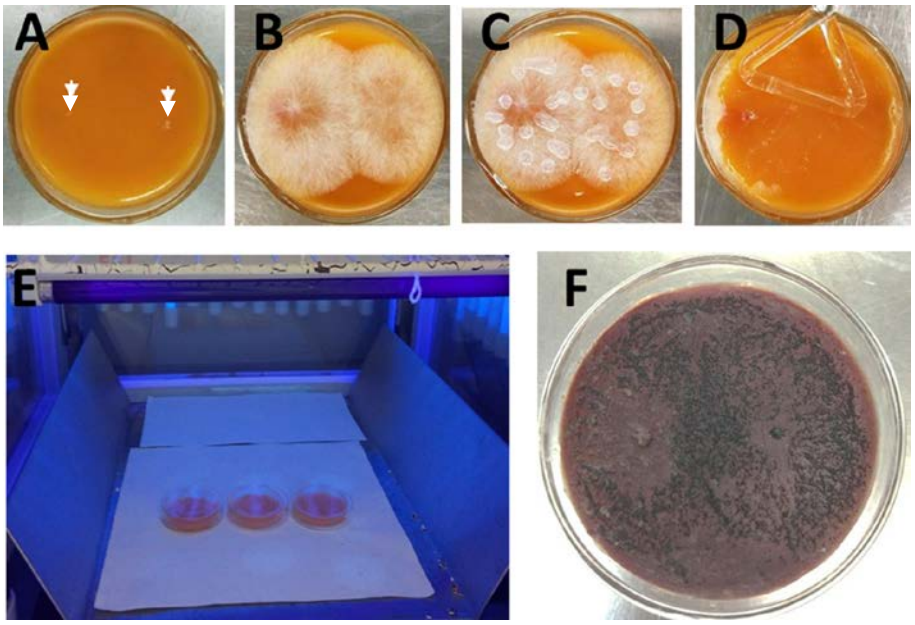

Figure 1. Cross of mutants and perithecia formation on carrot agar. A. Inoculate carrot agar with two gene mutants. The arrows indicate the inoculation sites. B. Two colonies met on the 3rd day after carrot agar inoculation. C. Add TWEEN 60 on the surface of carrot agar. D.

Press aerial mycelium down with a glass rod. E. The plate was placed under UV light in the chamber. F. Perithecia matured on the 7th day after adding TWEEN 60.

6. Scrape the perithecia along the intersection of two colonies and transfer to a 1.5 ml sterile centrifuge tube with sterile toothpicks (Figures 2A and 2B). Add 1 ml sterile ddH_2O to resuspend the perithecia and vortex at 1,000 rpm for 15 min to release the ascospores (Figure 2C).
7. Let the centrifuge tube stand for 2 min (Figure 2D) and transfer the supernatant including ascopores to a new centrifuge tube. Count the ascopores with a hemocytometer and adjust the concentration to 1 x 10^5 cells/ml.
   *Note: The concentration is dependent on the efficiency of recombination, the concentration can range from 1 x 10^4 cells/*ml *to 1 x 10^6 cells/*ml *for progeny screen in the next step.*
8. Spread 500 µl ascospores to 90 mm diameter TB3 plate supplemented with 400 mg/L hygromycin and 50 mg/L G418, incubate in a mold incubator at 25 °C in the dark for 3-4 days (Figure 2E).
9. Transfer the individual colonies to V8 juice agar with antibiotic (one colony is transferred to each separate plate). Place the plates at 25 °C for three days and then extract gDNA for further PCR verification (Figure 2F) as described in Nicholson (2007).

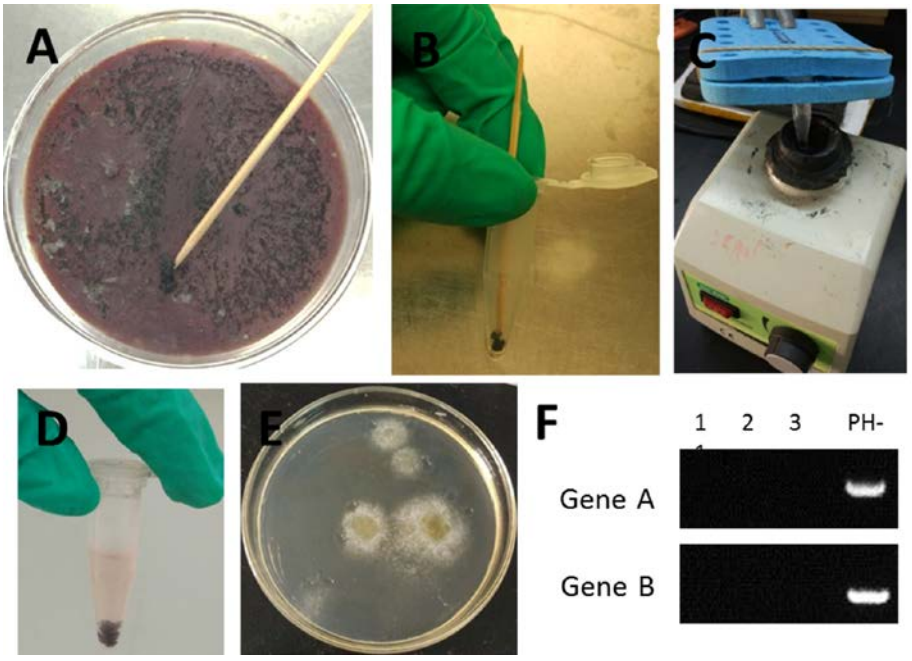

Figure 2. Recombinant progeny screening. A. The intersection of two colonies was scrapped with a toothpick. B and C. The perithecia were collected in centrifuge tubes (B), suspended in water and vortexed (C). D. Ascospores suspension. E. Single colonies grew on selective TB3. F. Genomic DNA PCR validation of double mutants.

B. Generation of triple mutants

1. Inoculate carrot agar plate with double mutants (A B mutants with *hph* and *npt*)) and the third gene mutants (C mutants with *nat*).
2. Repeat Steps A2-A7 to get the ascospore solution to a concentration of 1 x 10^6 cells/ml, then spread 500 µl ascospores on a 90 mm diameter TB3 plate supplemented with 400 mg/L hygromycin, 50 mg/L G418 and 50 mg/L nourseothricin, incubate in a mold incubator at 25 °C in the dark for 3-4 days.

   Note: The concentration of ascospores is also dependent on the efficiency of recombination, it ranges from 1 x 10^5 cells/ml to 1 x 10^7 cells/ml for progeny screen in the next step.
3. Transfer the individual colonies to V8 juice agar plate with antibiotics, culture the plates at 25 °C for three days and then extract DNA for further PCR verification with gene-specific primers as described in the data analysis section (Figure 3).

Note: There is a video presenting the method of generating recombinant perithecia of F. graminearum (https://www.jove.com/video/3895/sexual-development-and-ascospore-discharge-in-fusarium-graminearum). Our protocol was adapted from the video with modifications in fungal culture conditions, aerial mycelium press, perithecia induction conditions and ascospores discharge. In our protocol, we also improve the method of screening recombinants with antibiotics screening. The video can be a visual reference to this protocol.

Notes

1. Some mutants with defects in perithecia production may also be used to outcross with other mutants. Because these mutants may have defects in self-crossing, they could effectively outcross.
2. The decisive steps in perithecia production are A2 and A4 in Procedure section, so make sure the surface of the plate is smooth after pressing in Step A2 and check the plate every day after induction to avoid aerial mycelium growth in Step A4.
3. Use fresh carrot agar, the carrot agar stored too long can't produce perithecia.

Data analysis

The false positive rates in recombinant progeny screening for double mutants and triple mutants were found to be zero when we conducted the experiments (Figure 3). Therefore, this is a highly efficient method for generating double and triple mutants using the sexual crossing and antibiotic screening.

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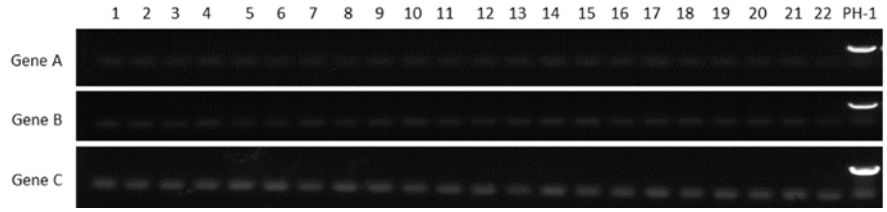

Figure 3. PCR validation of all recombinant progenies in the triple mutants screening. All individual colonies were found to be positive recombinant progeny and the false positive rate was zero. The used primers were to amplify a sequence within the targeted genes.

Recipes

1. Carrot Agar (1 L)
   a. Wash 400 g of fresh carrots, peel, and cut into cubes
   b. Put the cut carrot in one flask with 400 ml of water and autoclave at 121 °C for 15 min
   c. Homogenize with a homogenizer until the mixture appears smooth
   d. Add 500 ml of H_2O, mix well and disperse 200 ml into each 500 ml flask with 4 g agar (2% agar) and autoclave at 121 °C for 15 min
   e. Pour carrot agar into 60 mm diameter plates with 100 mg/L ampicillin

   Note: The carrot agar can be stored at 4 °C for up to one month.
2. 2.5% TWEEN 60 (100 ml)
   a. Weigh 2.5 g of semi-solid TWEEN 60 out into a 1.5 ml sterile centrifuge tube

      Note: The semi-solid TWEEN 60 is sticky. To be more precise, we added it to 100 ml H_2O with a centrifuge tube.
   b. Mix with100 ml H_2O and autoclave at 121 °C for 15 min
   c. Store at room temperature, and it is stable for years
3. TB3 plate (1 L)
   a. Weigh 3 g of yeast extract, 3 g of casamino acids, 20 g of sucrose
   b. Add distilled water and adjust the volume to 1 L
   c. Split the solution into 500 ml flasks each with 1.4 g agar in 200 ml solution, and then autoclave at 121 °C for 15 min
   d. Pour into 90 mm diameter plates with antibiotics for recombinant progeny screening
4. V8 juice agar (2 L)
   a. Weigh 2 g $CaCO_3$ and mix it with 340 ml V8 juice
   b. Add distilled water and adjust the volume to 2 L
   c. Split the solution into 500 ml flasks each with 3 g agar 200 ml solution, and then autoclave at 121 °C for 15 min
   d. Pour V8 juice agar into 60 mm diameter plates with antibiotics for V8 juice agar plate

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