

Generation of *Fusarium graminearum* Knockout Mutants by the Split-marker Recombination Approach

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[Abstract] *Fusarium graminearum* is a destructive phytopathogen and shows an impressive metabolic diversity. Gene deletion is an important and useful approach for gene function study. Here we present a protocol for generating gene deletion mutant by applying “split-marker” deletion strategy (Catlett *et al.*, 2003) with PEG-mediated protoplast transformation (Yuan *et al.*, 2008; Martín, 2015).

Keywords: *Fusarium graminearum*, Gene knock out, Split-marker recombination

Materials and Reagents

1. Pipette tips (Corning, Axygen[®], catalog number: T-300-R-S)
2. Sterile centrifuge tube (Corning, Axygen[®], catalog number: MCT-150-C)
3. PCR tubes (Corning, Axygen[®], catalog number: PCR-02-C)
4. 50 ml volume centrifuged tubes (Corning, Axygen[®], catalog number: SCT-50ML-25-S)
5. Scalpel (Fisher Scientific, catalog number: 08-920B)
6. 15 ml volume centrifuge tube (Corning, Axygen[®], catalog number: SCT-15ML-25-S)
7. Petri dish
8. Medical gauze (regular cotton yarn, Shanghai Honglong Medical Material Company)
9. Niex nylon membrane
10. Sterile toothpicks
11. Fungal strains: *F. graminearum* PH-1(NRRL 31084)
12. Primers (store at 4 °C for using within one month, store at -20 °C for using within six months)
 - a. Primer HY-F
 - b. Primer HY-R
 - c. Primer YG-F
 - d. Primer YG-R
 - e. Primer Up-F
 - f. Primer Up-R
 - g. Primer ID-F
 - h. Primer ID-R
 - i. Primer ID-2F

- j. Primer ID-2R
13. Ampicillin sodium Salt (Yeasen, catalog number: 60203ES60)
 14. Hygromycin B (Sigma-Aldrich, Roche Diagnostics, catalog number: 10843555001)
 15. Chloroform (Sinopharm Chemical Reagent, catalog number: 10006818)
 16. Isopropyl alcohol (Sinopharm Chemical Reagent, catalog number: 80109218)
 17. 75% ethanol (Sinopharm Chemical Reagent, catalog number: 80176961)
 18. Sterile ddH₂O
 19. AxyPrep™ DNA Gel Extraction kit (Corning, Axygen®, catalog number: AP-GX-250)
 20. KOD FX PCR kit (TOYOBO, catalog number: KOD-101)
 21. Agarose (Biowest, catalog number: 111860)
 22. TAE buffer (Thermo Fisher Scientific, catalog number: B49)
 23. Lysing enzymes (Sigma-Aldrich, catalog number: L1412)
 24. Driselase (Sigma-Aldrich, catalog number: D9515)
 25. Chitinase (Sigma-Aldrich, catalog number: C6137)
 26. KCl (Sinopharm Chemical Reagent, catalog number: 10016318)
 27. Ampicillin (Yeasen, catalog number: 60203ES10)
 28. Yeast extract (Thermo Fisher Scientific, Oxoid™, catalog number: LP0021B)
 29. Casamino acids (Sigma-Aldrich, catalog number: 22090)
 30. Sucrose (Sinopharm Chemical Reagent, catalog number: 10021418)
 31. CaCO₃ (Sinopharm Chemical Reagent, catalog number: 10005717)
 32. Tris (AMRESCO, catalog number: 0826)
 33. CaCl₂ (Sinopharm Chemical Reagent, catalog number: 10005861)
 34. PEG4000 (Sinopharm Chemical Reagent, catalog number: 30151626)
 35. NaCl (Sinopharm Chemical Reagent, catalog number: 10019318)
 36. EDTA (Sinopharm Chemical Reagent, catalog number: 10009617)
 37. Peptone (Sinopharm Chemical Reagent, catalog number: 10014963)
 38. D-glucose (Sinopharm Chemical Reagent, catalog number: 10010518)
 39. V8 juice (Campbell Soup Company)
 40. CTAB (Sinopharm Chemical Reagent, catalog number: 30037416)
 41. Plasmid pUCATPH (the sequence can be download from website: http://www.snapgene.com/resources/plasmid_files/yeast_plasmids/pUCATPH/)
 42. Genomic DNA preparation of *F. graminearum* in ddH₂O
 43. 0.22 µm PES membrane (Merck, catalog number: GPWP14250)
 44. YEPD liquid medium (see Recipes)
 45. Mycelium enzymolysis mixture (see Recipes)
 46. STC buffer (see Recipes)
 47. PTC (see Recipes)
 48. TB3 plate (see Recipes)
 49. Low-melting-temperature TB3 (see Recipes)

50. V8 juice agar (see Recipes)
51. 1.5x CTAB (see Recipes)
52. Mung bean liquid medium (see Recipes)
53. 1.2 M KCl (see Recipes)

Equipment

1. Hemocytometer (0.10 mm, 1/400 mm²) (QIUJING, model: XB-K-25)
2. Flask (SiQi, model: SP250SJ)
3. Electronic balance (OHAUS, model: AR1140)
4. Mold incubator (Yiheng, model: MJ-150-I)
5. Micropipettes (Eppendorf)
6. Gel electrophoresis chamber (Tanon, model: EPS300)
7. Gel apparatus (Helixx Mupid-exU) and image system (Tanon gel image system, model: 1600)
8. Microcentrifuge (Eppendorf, model: 5418, catalog number: 022620304)
9. Centrifuge (Beckman Coulter, model: Avanti[®] J-E)
10. PCR system (Bio-Rad Laboratories, model: S1000)
11. Orbital shaker (Kylin-Bell Lab Instruments, model: TS-2)
12. Shaker (Boyn Industria, model: THZ-C-1)
13. Biological safety cabinet (ESCO Micro, model: FHC1200A)
14. Microscope (Olympus, model: BX51)
15. Autoclave (SANYO, model: MLS-3781L-PC)
16. Ultra-low Temperature Freezer (Thermo Fisher Scientific, model: TSE400V)
17. Tube holder (LUOBENDE)
18. Tissuelyser II (QIAGEN, model: 85300)
19. Growth chamber (JIANGNAN INSTRUMENT, model: RXZ-1000)
20. DuoFlow pH Monitor (Bio-Rad Laboratories, model: 760-2040)

Procedure

For generating single gene deletion mutants, a variety of genes conferring resistance to antibiotics are available, *e.g.*, genes resistant to Hygromycin B, Geneticin/G418, Bialaphos/Phosphothricin, Nourseothricin, Blasticidin, and Phleomycin. Among these, the gene resistant to Hygromycin B is the most widely used. In this protocol, we use *hph* as the resistant gene. Other markers can also be chosen according to your experiment as well (Turgeon *et al.*, 2010).

A. PCR fusion

1. Primers design (Figure 1A)
 - a. Download the *hph* unit sequence (contains promoter, *hph*, and terminator sequence) and

- design four primers, Primers HY-F and HY-R amplify the 5' half of *hph* unit (named HY), YG-F and YG-R amplify the 3' half of *hph* unit (named YG). The overlap between HY and YG fragments should be at least 300 bp.
- Download the upstream 1 kb and downstream sequence 1 kb of the target gene and design four gene-specific primers—Primers Up-F and Up-R, Down-F and Down-R. Primers Up-F and Up-R amplify the upstream fragment (about 1 kb), Down-F and Down-R amplify the downstream fragment (about 1 kb) (Figure 1A).
 - An adaptor sequence which is the reverse complementary sequence of HY-F is added at the 5' of Up-R (Table 1) and an adaptor sequence which is the reverse complementary sequence of YG-R is added at the 5' of Down-F (red letter).

Table 1. Primer sequences

Primer name	Sequences	Sequences match
HY-F	GCGGCTTCGAATCGTGGCTA	promoter region of <i>hph</i> unit
HY-R	GTATTGACCGATTCCCTTGCGGTCCGAA	terminator region of <i>hph</i> unit
YG-F	GATGTAGGAGGGCGTGGATATGTCCT	<i>hph</i> sequence
YG-R	CATACTCTTCCTTTTCAATTCAATTCAATGCG	<i>hph</i> sequence
UP-F	20bp of 5' upstream of the target gene	upstream region of the target gene -1kb before ATG
UP-R	TAGCCACGATTCGAAGCCGC + 20bp of target gene before ATG region	upstream region of the target gene -1kb before ATG
DOWN-F	CGCATTGAATTGAAAAAGGAAGAGTATG + 20bp of target gene after TAA/TAG/TGA	downstream region of the target gene -1kb after TAA/TAG/TGA
DOWN-R	20bp of 3' downstream of the target gene	downstream region of the target gene -1kb after TAA/TAG/TGA

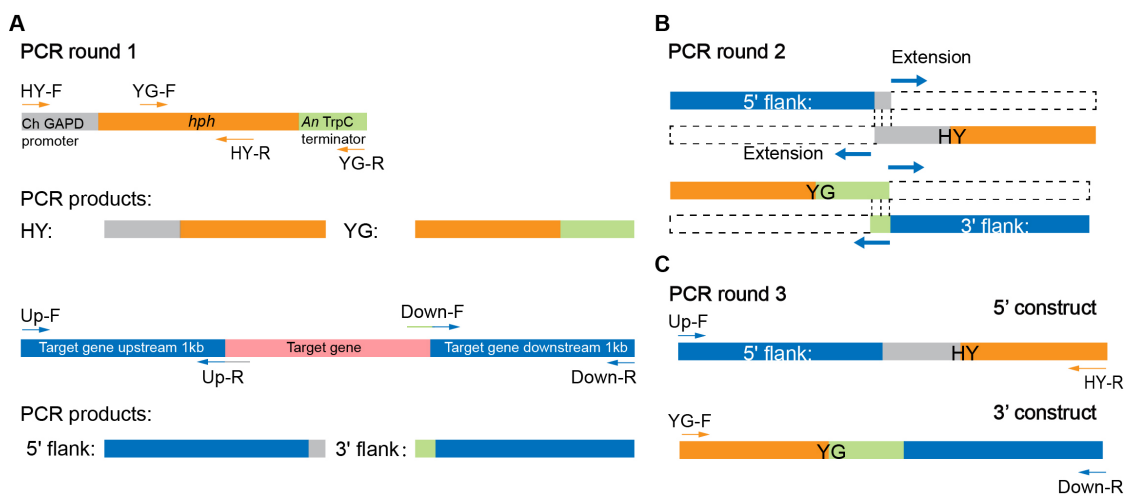


Figure 1. Diagram of PCR fusion process. A. Primers and amplification product of PCR round 1. B. PCR round 2, each flank from round 1 is fused to the marker through PCR by overlap extension. C. PCR round 3, amplification of the products from round 2 with primers.

2. PCR round 1

In PCR round 1 (Figure 1A), the flanks and the selectable marker *hph* gene are amplified individually (four reactions). The overlapping fragments "HY" and "YG" of the hygromycin phosphotransferase cassette are amplified from template plasmid pUCATP H (0.5 µg/µl) (Catlett *et al.*, 2003). The genomic DNA (0.1 µg/µl) for *F. graminearum* is used as the template for 5' flank and 3' flank amplification.

50 µl PCR reaction mixture:

2x KOD buffer (provided in the PCR enzyme kit)	25 µl
H ₂ O	18 µl
2 mM dNTP (provided in the PCR enzyme kit)	5 µl
1 µM Primer 1	2 µl
1 µM Primer 2	2 µl
KOD FX	1 µl
Template	2 µl

PCR program:

Stage 1	94 °C	4 min
Stage 2	94 °C	30 sec
	50 °C	30 sec
	68 °C	60 sec
35 cycle		
Stage 3	68 °C	10 min

Prepare a 1% agarose gel and analyze the PCR products by electrophoresis. Recover the target DNA fragments (HY, YG, 5' flank and 3' flank, Figure 1A) using a scalpel and purify using AxyPrep™ DNA Gel Extraction kit.

3. PCR round 2

In PCR round 2 (Figure 1B), each flank from round 1 is fused to the marker through PCR by overlap extension (two reactions).

For the 5' construct, templates are the HY-F/HY-R marker fragment ("HY") (Template 1) and F1/R1 flank (Template 2).

For the 3' construct, templates are the YG-F/YG-R marker fragment ("YG") (Template 3) and F2/R2 flank (Template 4).

50 µl PCR reaction mixture:

2x KOD buffer	25 µl
2 mM dNTP	5 µl
KOD FX	1 µl
100~200 ng/µl Template 1/3	9.5 µl
100~200 ng/µl Template 2/4	9.5 µl

PCR program:

Stage 1	94 °C	4 min
Stage 2	94 °C	30 sec
	50 °C	30 sec
	68 °C	2 min
10 cycle		
Stage 3	68 °C	10 min

Note: No primers are included in this round.

4. PCR round 3

In PCR round 3 (Figure 1C), the templates are the products of PCR round 2. For the 5' construct, templates are the PCR product of Template 1/2, primers are F1 and HY-R. For the 3' construct, templates are the PCR product of Template 3/4, primers are YG-F and R2.

50 µl PCR reaction mixture:

2x KOD buffer	25 µl
H ₂ O	18 µl
2 mM dNTP	5 µl
1 µM Primer 1	2 µl
1 µM Primer 2	2 µl
KOD FX	1 µl
100~200 ng/µl Template	2 µl

PCR condition:

Stage 1	94 °C	4 min
Stage 2	94 °C	30 sec
	50 °C	30 sec
	68 °C	2 min
35 cycle		
Stage 3	68 °C	10 min

Analyze the PCR products by agarose gel electrophoresis, recover the target DNA fragments and purify using AxyPrep™ DNA Gel Extraction kit.

B. Protoplasts preparation

1. *F. graminearum* conidia were cultured in mung bean liquid medium and collected as described in Procedure B of Jia *et al.*, 2017. Transfer all collected conidia into a flask with 250 ml fresh sterilized YEPD liquid medium.
2. Incubate in a constant temperature shaker at 25 °C and 175 rpm for 12-14 h.
Note: Incubation time should be no more than 14 h.
3. Prepare the mycelium enzymolysis mixture as described in Recipe 2.
4. Filter the YEPD liquid medium culture as shown in Figure 2A. Then wash the gauze with the enzyme mixture (30 ml) and transfer all the mycelium into a 100 ml flask.
5. Incubate in a constant temperature shaker at 30 °C and 90 rpm.
6. Observe protoplasts under a microscope every hour until most mycelia have been digested exhibiting spherical shape (Figure 2B). Usually, it takes about 2 h.
7. Filter the enzymolysis mixture through Niex nylon membrane and collect about 25 ml filtrate into a 50 ml centrifuge tube (Figure 2C), then centrifuge the tube at 1,100 x g for 5 min at room temperature. Pour out the supernatants slowly.
8. Resuspend the protoplasts sedimentation with approximately 3 ml 1.2 M KCl in the 50 ml centrifuge tube. Pipet 50 µl onto a hemocytometer to calculate protoplasts concentration under the microscope. The expected range of concentration is from 1 x 10⁶/ml to 1 x 10⁸/ml. Then centrifuge the tube at 1,100 x g for 5 min again, remove the supernatants with a pipette.
Note: The volume of KCl used here can be increased or reduced based on the production volume of the protoplasts sedimentation.
9. Resuspend protoplasts sedimentation gently in STC to the final concentration of 2-5 x 10⁷/ml (Figure 2D).
10. Aliquot protoplast suspension into sterile centrifuge tubes by 200 µl/tube and store in a -70 °C Ultra-low Temperature Freezer.

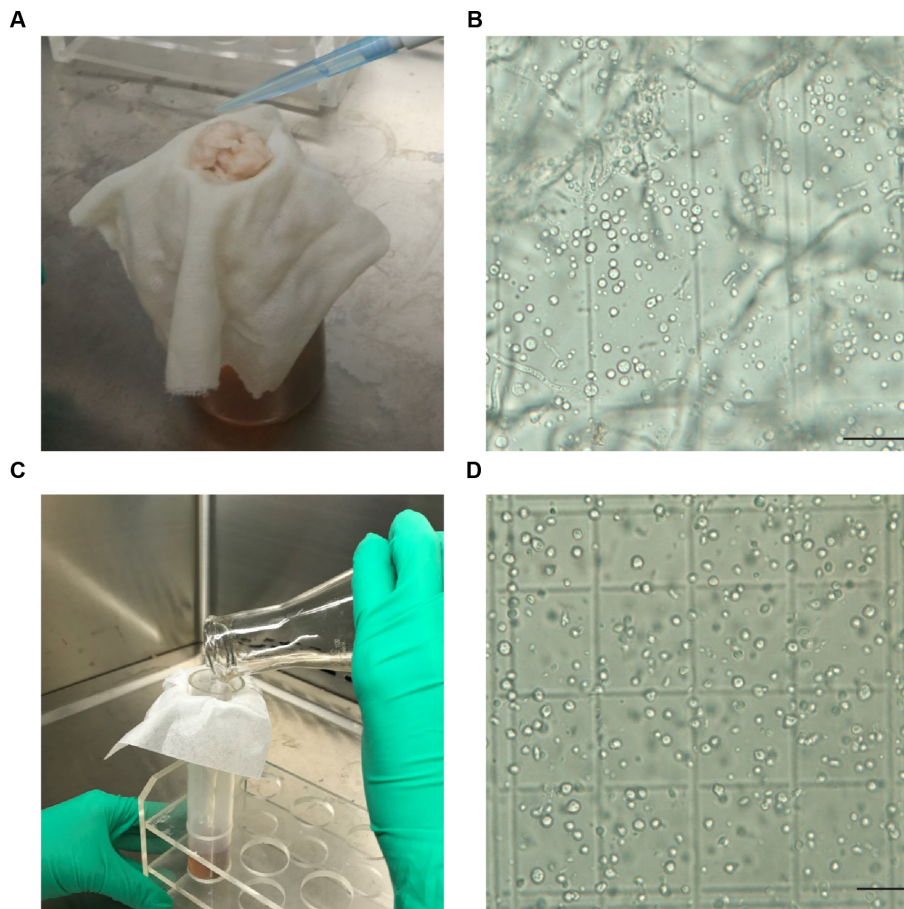


Figure 2. Protoplasts preparation. A. Filter the YEPD liquid medium culture and wash the gauze with the enzyme mixture. B. Pipette 10 μ l protoplast mixture 2 h after incubation and observe protoplast formation under the microscope. C. Filter the enzymolysis mixture through Niex nylon membrane. D. Dissolve protoplasts in STC, then count its concentration with a hemocytometer and adjust the final concentration to $2-5 \times 10^7$ /ml. Scale bars = 20 μ m.

C. Transformation of protoplasts

1. Thaw the protoplasts on ice. Melt Low-melting-temperature TB3 in advance and keep the temperature at 37 °C.
2. Add the PCR products (> 200 ng/ μ l) gained in Procedure A to the protoplasts and transfer into a sterile 15 ml volume centrifuge tube. Mix protoplasts and DNA fragments gently then set on a tube holder at room temperature for 15 min.
3. Add 100 μ l PTC and gently mix.
4. After 1 min, add 200 μ l PTC and gently mix.
5. After 1 min, add 700 μ l PTC and gently mix, and then set on the tube holder for 13 min (Figure 3A).
6. Add 5 ml Low-melting-temperature TB3, invert the tube at least three times and pour it onto the top of the TB3 plate supplemented with ampicillin and hygromycin B (Figures 3B and 3C).

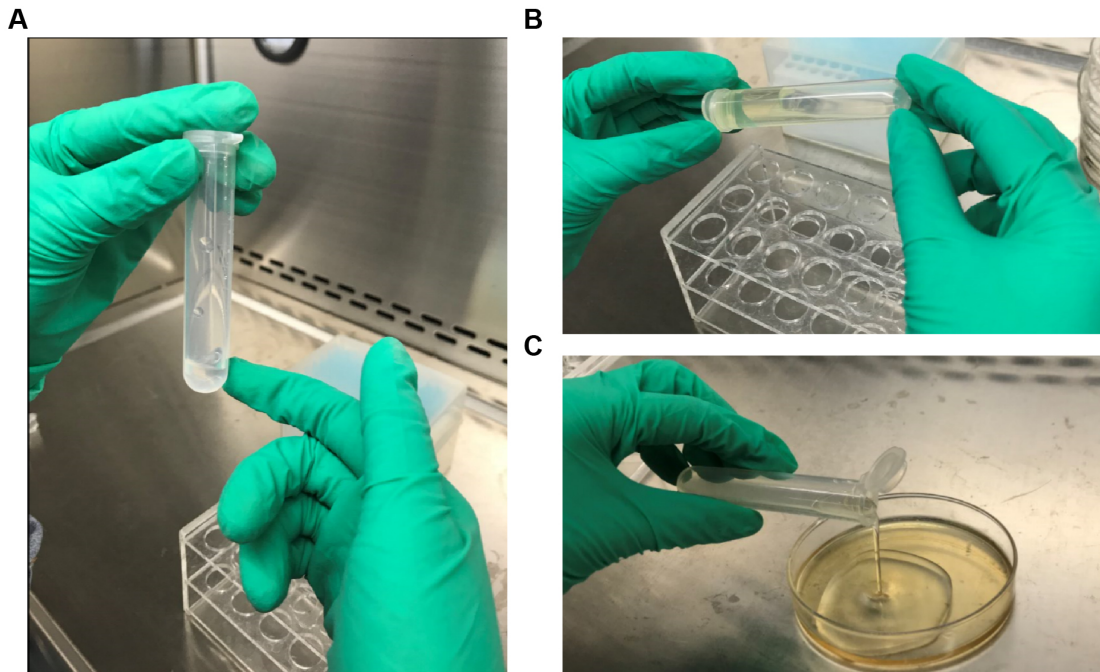


Figure 3. Transformation of protoplasts. A. Add PCR products and PTC and mix gently. B. After addition of low-melting-temperature TB3, invert the tube at least three times. C. Pour the mixture onto the top of the TB3 plate and incubate the plate in a 25 °C growth chamber for 3 days.

D. Screen and verification knock-out mutant

1. Incubate the transformed TB3 plate in a 25 °C growth chamber for 3 days (Figure 4A).
2. Pick the edge mycelia of transformants using a sterile toothpick, and transfer to a new TB3 plate supplemented with ampicillin and hygromycin B to get a series of single colonies. Incubate in a 25 °C growth chamber for 1 day.
3. Pick the mycelia of each colony and transfer to a fresh V8 agar plate. Incubate the culture in a 25 °C growth chamber for 3 days. (Figure 4B)

Note: Before next step, it is recommended to stock a copy of the colony in slant V8 culture-medium.

4. Scrape mycelia from V8 plate into a centrifuge tube and add 350 µl of 1.5x CTAB for genomic DNA extraction.
5. Grind the sample in the tissuelyser, then incubate in the 65 °C water bath for 30 min, shaking them gently every 10 min. Add 350 µl of chloroform and shake the tubes gently. Sit the tubes on the bench until they cool to room temperature.
6. Pipet the 200 µl upper phase to new tubes. Add 200 µl of isopropyl alcohol and shake the tubes gently. Spin the tubes for 10 min at 12,000 x g.
7. Discard the supernatant and reserve the pellet, then wash the sediment with 500 µl 75% Ethanol. Spin the tubes for 2 min at 12,000 x g.
8. Remove the 75% Ethanol and very gently tap the tube over a paper towel.
9. Air dry on the bench for 10-15 min or until dry. Dissolve the pelleted DNA in TE (pH 8.0) or

deionized water.

10. Verify gene deletion mutants by two PCR reactions using genomic DNA as the template and the primers described below (Figures 4C and 4D).
 - a. Reaction 1: two primers outside of the target gene—ID1F upstream of the target gene, ID1R downstream of the target gene.
 - b. Reaction 2: two primers inside of the target gene—ID2F/ID2R.
 - c. A correct knockout mutant should have:
 - no amplification products produced with primers ID2F/2R;
 - target gene fragments with expected size produced with primers ID1F/1R, as shown in Figure 4C.

Note: PCR with wild-type Fusarium graminearum PH-1 genomic DNA should be included as a positive control. ID2F/2R can amplify the fragment inside the target gene.

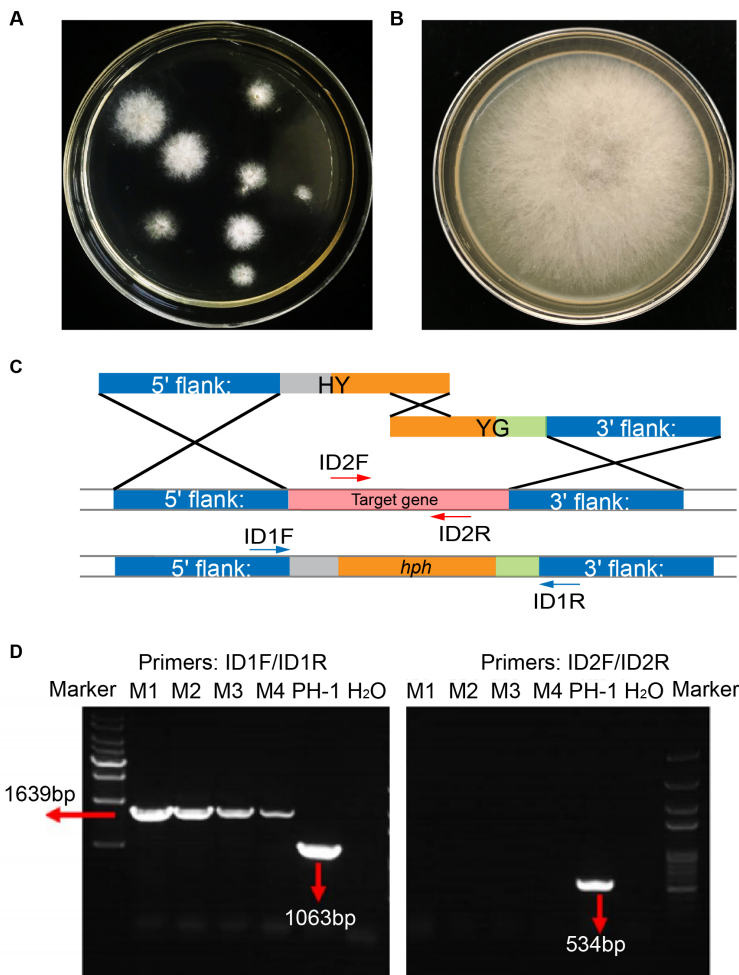


Figure 4. Screen and verification knock-out mutant. A. The transformed TB3 plate incubation in 25 °C growth chamber for 3 days. B. The V8 plate with single colony after incubated in 25 °C growth chamber for 3 days. C. The diagram of principle of knock out and strategy of identification PCR. D. PCR validation of knock-out mutants.

Notes

1. This protocol has been proven to be suitable for multiple gene knockouts. Not only knock out certain fragments of a gene, but also can knock out a gene cluster that contains multiple genes.
2. The size of DNA region knockout ranges from 300 bp to 2 kb. The longest region we have successfully knocked out with this protocol is about 37 kb. However, as the length of the knockout fragment increases, the probability of successful gene knockout gradually decreases and the upstream and downstream homologous regions also need to be longer than 1 kb.
3. If you want to knock out a single gene, we recommend using resistance gene *hph* and using amplification primers we provided in Table 1.

Recipes

1. YEPD liquid medium

Yeast extract	3.0 g/L
Peptone	10.0 g/L
D-glucose	20 g/L

2. Mycelium enzymolysis mixture

Lysing enzymes	450 mg
Driselase	750 mg
Chitinase	12 mg

Dissolve in 30 ml 1.2 M KCl and centrifuge at 8,000 x *g* for 8 min. Because the enzymes are not fully soluble, you should discard the sediment and reserve the supernatant.

3. STC buffer (pH = 8.0)

Sucrose	20%
Tris-HCl	10 mM
CaCl ₂	50 mM

4. PTC

PEG4000 40%

Dissolve in STC and filter to remove bacteria through 0.22 μm PES membrane

5. TB3 plate

Yeast extract	3 g
Casamino acids	3 g
Sucrose	20%
Agar	0.7%

dH₂O to reach 1 L

Autoclave at 121 °C for 15 min

Pour the medium into Petri dishes

6. Low-melting-temperature TB3

Yeast extract	3 g
Casamino acids	3 g
Sucrose	20%
Agar	0.7%

dH₂O to reach 1 L
Autoclave at 121 °C for 15 min
7. V8 juice agar

V8 juice	340 ml
CaCO ₃	2 g
Agar	1.5%

dH₂O to reach 2 L
Autoclave at 121 °C for 15 min
8. 1.5x CTAB

CTAB	15 g/L
NaCl	61.4 g/L
0.5 M EDTA	30 ml/L
1 M Tris-HCl (pH = 8.0)	75 ml/L
9. Mung bean liquid medium (1 L)
 - a. 40 g mung bean (*Vigna radiata*) (dried, available at grocery stores or supermarket)
 - b. Put the mung bean in the boiling water, then boil for about 10 min and let it cool to room temperature
 - c. Filter through gauze and discard the bean residue
 - d. Add up to 1 L with distilled water
 - e. Autoclave at 121 °C for 20 min
10. 1.2 M KCl

KCl	89.46 g/L
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Autoclave at 121 °C for 20 min

Acknowledgments

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