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## Filamentous Fungi (*Magnaporthe grisea* and *Fusarium oxysporum*)

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**Summary**

A better understanding of fungal biology will facilitate judicious use of beneficial fungi and will also advance our efforts to control pathogenic fungi. Molecular studies of fungal biology have been greatly aided by transformation-mediated mutagenesis techniques. Transformation via nonhomologous integration of plasmid DNA bearing a selectable marker (e.g., antibiotic resistance gene) has been widely used for the random insertional mutagenesis of fungi—as an alternative to chemical and radiation mutagens—mainly because the integration of plasmid into the genome provides a convenient tag for subsequent identification and isolation of the mutated gene. Homologous recombination between a target gene on the chromosome and the introduced DNA carrying its mutant allele results in targeted gene knock-out. An important advance in fungal transformation methodology is the development of *Agrobacterium tumefaciens*-mediated transformation (ATMT) protocols for fungi. ATMT has been successfully applied to a phylogenetically diverse group of fungi and offers a number of advantages over conventional transformation techniques in both the random insertional mutagenesis and targeted gene knock-out. In this chapter, we describe ATMT protocols and vectors for fungal gene manipulation using two plant pathogenic fungi, *Magnaporthe grisea* and *Fusarium oxysporum,* as target organisms.

**Key Words:** *Agrobacterium tumefaciens*; binary vector; conditional negative selection; *Fusarium oxysporum*; gene knock-out; herpes simplex virus thymidine kinase; *Magnaporthe grisea*; transformation.

### 1. Introduction

Transformation-mediated forward and reverse-genetic analyses have greatly facilitated functional studies of fungal genes *(1)*. In most filamentous fungi, transformation results from the integration of introduced DNA into the fungal genome by either nonhomologous or homologous recombination. Mutagenesis

From: *Methods in Molecular Biology, vol. 344:* Agrobacterium *Protocols, 2/e, volume 2*
Edited by: Kan Wang © Humana Press Inc., Totowa, NJ

of fungi through random integration of the transforming DNA via nonhomologous recombination has been widely used for tagging and isolating fungal genes that are involved in various aspects of fungal biology *(2–5)*. Mutagenesis of a specific gene via homologous recombination between the target gene and its mutant allele that has been introduced through transformation is also routinely performed in many fungi. A typical procedure for transforming filamentous fungi involves the preparation of fungal protoplasts, delivery of the transforming DNA with associated selectable marker, and selection of the generated transformants. The protoplast generation step, which involves the digestion of the fungal cell walls using a mixture of hydrolytic enzymes, is often critical for high-transformation efficiency and is difficult to optimize for reproducibly producing good protoplasts. *Agrobacterium tumefaciens*–mediated transformation (ATMT) offers an alternative and versatile means for transforming fungi without relying on protoplasts.

Since the demonstration of successful T-DNA transfer into *Saccharomyces cerevisiae* by *A. tumefaciens* *(6)*, ATMT has been successfully applied to transform a diverse array of fungi *(7–24)*. For successfully transforming fungi, *A. tumefaciens* appears to utilize the same virulence genes that are required for plant transformation *(6,25)*, suggesting that the bacterium utilizes a conserved mechanism for transformation regardless of hosts. In addition to the ability to transform spores, hyphae, and even mushroom fruiting body tissue, ATMT exhibits a number of other advantages as a method for forward and reverse genetic analyses of fungi: (1) high transformation efficiency, resulting in several hundred transformants per $1 \times 10^6$ spores in many fungal species *(12,19,20)*, (2) increased frequency of homologous recombination, a feature conducive for efficient targeted gene knock-out *(13,24,26)*, and (3) low-copy-number of inserted T-DNA per genome (less than two on average), which facilitates the identification of a gene tagged by the T-DNA especially in asexual fungi *(12,19–21)*.

Although targeted gene knock-out via transformation is feasible in many fungi, for fungi that exhibit low frequencies of homologous recombination, identification of the desired mutant often requires that a large number of transformants be generated and screened *(14,27)*. To circumvent this time-consuming process, a new targeted gene knock-out method, which is based on ATMT with a mutant allele of the target gene flanked by a conditional negative selection marker, was developed (*see* **Fig. 1**). A dual (positive and negative) selection of transformants permitted the enrichment of target mutants *(14,27)*. This method, termed ATMT-DS, can potentially be applied to a broad spectrum of fungi and may serve as a powerful functional genomic tool in fungal research. In this chapter, protocols and vectors for ATMT and targeted mutagenesis of filamentous fungi are described using two plant pathogenic fungi, *Magnaporthe grisea*

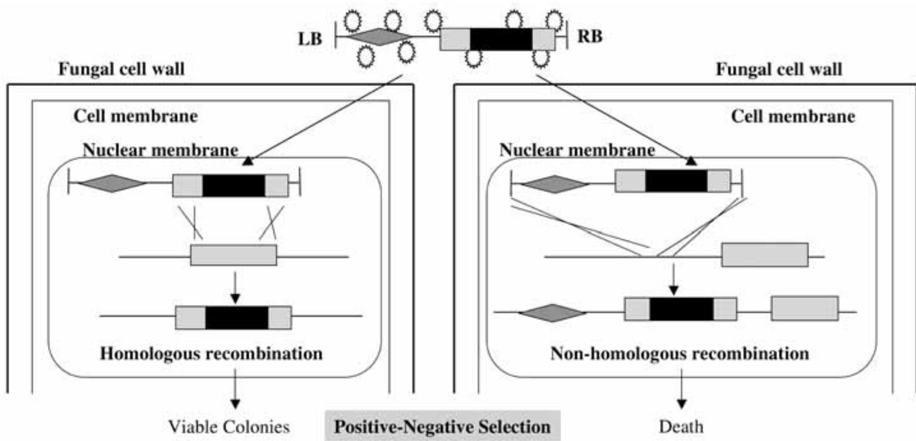


**Fig. 1.** Schematic diagram of ATMT of fungi and ATMT-DS. *Agrobacterium tumefaciens* cells, carrying a binary vector that contains a mutant allele (disrupted by a positive selection maker, such as *hpt* conferring resistance to hygB; marked as the filled box) and *HSVtk* (encoding herpes simplex virus thymidine kinase that converts nucleoside analogs such as 5-fluoro-2′-deoxyuridine [F2dU] to a compound toxic to fungi; denoted by the diamond) on the T-DNA, are co-incubated with fungal cells in the presence of AS, a chemical inducer of virulence genes of *A. tumefaciens*. During co-cultivation, DNA situated between the left border (LB) and right border (RB) of the T-DNA is transported into fungal nuclei (probably as a complex with certain *A. tumefaciens* Vir proteins which were denoted by the circles). Homologous recombination between the chromosomal gene and the mutant allele on the T-DNA leads to the loss of *HSVtk*. If the T-DNA integrates into a random location in the fungal genome via non-homologous recombination, both *hpt* and *HSVtk* will be expressed. Gene knock-out mutants can be selected by subjecting transformants to both the positive (hygB) and negative (F2dU) selection agents.

and *Fusarium oxysporum*, as target organisms. In both fungi, we typically obtain between 100 and 400 transformants per $1 \times 10^6$ spores. These tools can also be applied to other fungi with no or little modification.

## 2. Materials

### *2.1. Fungal and Bacterial Strains* (see *Note 1)*

1. *M. grisea* KJ201 ***(28)***: An isolate from infected rice.
2. *M. grisea* 4091-5-8 ***(29)***: A laboratory strain derived from a genetic cross between two field isolates.
3. *F. oxysporum* strain O-685 ***(19)***: An isolate from cabbage.
4. *A. tumefaciens* strains AGL1 and EHA105 ***(30)***.
5. *Escherichia coli* strain XL1-Blue MRF (Stratagene, La Jolla, CA).

### 2.2. Culture Media

All culture media use 1.5% agar (Difco, Sparks, MD) to solidify medium and are autoclaved (120°C for 15 min) before use.

1. Oatmeal agar: After incubating 50 g of rolled oats (Quaker Oats, Chicago, IL) in 500 mL at 70ºC for 1 h, filter them through a cheesecloth, add agar, and bring the volume to 1 L.
2. Complete medium: 6 g of yeast extract, 6 g of casein acid hydrolysate, 10 g of sucrose/L.
3. 2YEG: 2 g of yeast extract and 10 g of glucose/L.
4. Carnation leaf agar: After sterilizing 1.5% agar in water by autoclaving, cool to 60°C. Pour agar solution in 90 mm diameter Petri plates containing sterilized (via irradiation) carnation leaf pieces (20–30 pieces/plate) (*see* **Note 2**).
5. Potato dextrose: 24 g of potato dextrose/L (Difco, Sparks, MD).
6. *Aspergillus* minimal medium: For 1 L of medium, add 50 mL stock salt solution (60 g $NaNO_3$, 5.2 g KCl, 5.2 g $MgSO_4{\cdot}7H_2O$ and 8.2 g $KH_2PO_4$/L), 10 g glucose, and 2 mL Hutner's trace elements (2.2 g $ZnSO_4{\cdot}7H_2O$, 1.1 g $H_3BO_3$, 0.5 g $MnCl_2{\cdot}4H_2O$, 0.5 g $FeSO_4{\cdot}7H_2O$, 0.16 g $CoCl_2{\cdot}6H_2O$, 0.16 g $CuSO_4{\cdot}5H_2O$, 0.11 g $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ and 5 g $Na_2$EDTA/100 mL).
7. CMC broth: 15 g of carboxymethyl cellulose, 1 g of yeast extract, 0.5 g of $MgSO_4$, 1 g of $NH_4NO_3$, and 1 g of $KH_2PO_4$/L (*see* **Note 3**).
8. Water agar: Autoclave 1.5% agar in water.
9. Luria–Bertani broth (LB): 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl/L.
10. Minimal medium (MM): *See* **Table 1** and **Note 4**.
11. Induction medium (IM): *See* **Table 1** and **Note 5**.
12. Co-cultivation medium (CM): *See* **Table 1** and **Note 5**.

### 2.3. Antibiotics and Other Chemical Agents (see *Note 6*)

1. Carbenicillin: Prepare as a stock of 100 mg/mL in $H_2O$.
2. Kanamycin: Prepare as a stock of 50 mg/mL in $H_2O$.
3. Rifampicin: Prepare as a stock of 10 mg/mL in methanol.
4. Acetosyringone (AS) (3,5-dimethoxy-4-hydroxyacetophenone): Prepare as a stock of 200 m*M* in 95% ethanol (*see* **Note 7**).
5. Hygromycin B (hygB): Prepare as a stock of 100 mg/mL in $H_2O$.
6. Cefotaxime, sodium salt: Prepare as a stock of 200 m*M* in $H_2O$.
7. Moxalactam, sodium salt: Prepare as a stock of 100 mg/mL in $H_2O$.
8. F2dU (5-fluoro-2′-deoxyuridine): Prepare as a stock of 10 m*M* in $H_2O$.
9. 1 M MES: dissolve 42.64 g of MES [2-(*N*-morpholino)ethanesulfonic acid] in 160 mL of deionized $H_2O$. The pH is adjusted to 5.3 with 10 *N* NaOH with stirring vigorously on a magnetic stirrer until the MES has completely dissolved. Then, deionized $H_2O$ is added to bring the final volume to 200 mL (*see* **Note 8**).

### 2.4. Binary Vectors for Fungal Transformation (see *Note 9 and Fig. 2*)

1. pBHt2: A vector carrying the *hpt* (hygB phosphotransferase) gene under the control of the *Aspergillus nidulans TrpC* promoter on the T-DNA.

**Table 1**
**Preparation of Stock Solutions and Transformation Media**

| | Stock solution (100 mL)[a] | | MM[b] | IM[c] | CM[d] |
|---|---|---|---|---|---|
| Reagent | Chemical | Amount required | Amount required to make 100 mL | | |
| K-buffer (pH 7.0) | $K_2HPO_4$ | 20 g | 1 mL | 1 mL | 1 mL |
| | $KH_2PO_4$ | 14.5 g | | | |
| M-N | $MgSO_4{\cdot}7H_2O$ | 3 g | 2 mL | 2 mL | 2 mL |
| | NaCl | 1.5 g | | | |
| 1% $CaCl_22H_2O$ | $CaCl_2{\cdot}2H_2O$ | 1 g | 0.1 mL | 0.1 mL | 0.1 mL |
| Spore elements | $ZnSO_4{\cdot}7H_2O$ | 0.01 g | 1 mL | 1 mL | 1 mL |
| | $CuSO_4{\cdot}5H_2O$ | 0.01 g | | | |
| | $H_3BO_3$ | 0.01 g | | | |
| | $MnSO_4{\cdot}H_2O$ | 0.01 g | | | |
| | $Na_2MoO_4{\cdot}2H_2O$ | 0.01 g | | | |
| 20% $NH_4NO_3$ | $NH_4NO_3$ | 20 g | 0.25 mL | 0.25 mL | 0.25 mL |
| 20% Glucose | Glucose | 20 g | 1 mL | 1 mL | 1 mL |
| 0.01% $FeSO_4$ | $FeSO_4$ | 0.01 g | 1 mL | 1 mL | 1 mL |
| 50% Glycerol | Glycerol | 50 mL | – | 1 mL | 1 mL |
| 1M MES (pH 5.3) | MES | 21.32 g | – | 4 mL | 4 mL |
| Sterile $H_2O$ | | | 93.5 mL | 88.5 mL | 88.5 mL |
| Kanamycin stock | | | 0.15 mL | 0.15 mL | 0.15 mL |
| Acetosyringone | | | – | 0.2 mL | 0.2 mL |
| Agar | | | – | – | 1.5 g |

[a]Prepare all the stock solutions (except AS) in deionized $H_2O$ as indicated. It is not necessary to sterilize the acetosyringone stock. Glucose, $FeSO_4$, kanamycin, and MES are sterilized by filtration through a 0.22-µm filter, and the remaining reagents are sterilized by autoclaving for 20 min at 120°C. Store them at 4°C except $FeSO_4$, kanamycin, acetosyringone, and MES (stored at –20°C).

[b]Minimal medium (*see* **Note 4**).

[c]Induction medium (*see* **Note 5**).

[d]Co-cultivation medium. CM is identical to IM except the addition of 1.5% agar.

2. pKHt: A vector carrying the *hpt* gene under the control of the *A. nidulans TrpC* promoter plus the ColE1 replication of origin and the chloramphenicol resistance gene on the T-DNA.
3. pDHt: A vector for the targeted mutagenesis of fungal genes via homologous recombination.
4. pGKO1: A vector for the targeted mutagenesis of fungal genes via ATMT-DS.
5. pGKO2: A vector for the targeted mutagenesis of fungal genes via ATMT-DS.

### *2.5. Other Solutions and Supplies*

1. 2-mL Cryostorage tubes.
2. 80% sterilized glycerol.

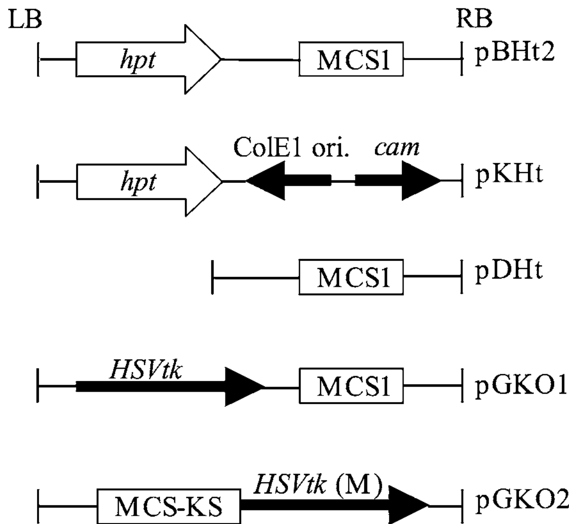


**Fig. 2.** Schematic diagrams of the T-DNA region in five binary vectors for fungal transformation. The LB and RB of the T-DNA are denoted by vertical lines. The orientation of transcription from *hpt, cam* (conferring resistance to chloramphenicol), and *HSVtk* is indicated by arrow (5′ to 3′). ColE1 ori indicates the ColE1 replication origin. MCS1 corresponds to the multiple cloning site of pCAMBIA1300 (offering nine unique restriction sites in pBHt2 and pDHt). In pGKO1, due to the presence of one or more of the *Bam*HI, *Pst*I, *Sac*I, *Sal*I, and *Sma*I site in *HSVtk*, only four restriction sites in MCS1 can be used for cloning a mutant allele. The multiple cloning site of pGKO2 (designated as MCS-KS) was derived from pGreenII0000 ***(34)*** and offers 15 unique restriction sites. The *Bam*HI, *Eco*RV, *Pst*I, *Sac*I, *Sal*I, and *Sma*I sites that are present in *HSVtk* were removed via site-directed mutagenesis to produce *HSVtk* (M). Drawings are not to scale.

3. 3-mm Sterilized soda lime glass beads (*see* **Note 10**).
4. Hemocytometer.
5. Cheesecloth.
6. 47-mm Nitrocellulose membrane.
7. Pasteur pipet (melt the tip in flame to produce a round bulb).
8. Genomic DNA extraction buffer: 50 m*M* Tris-HCl, pH 7.5; 100 m*M* ethylenediamine tetraacetic acid (EDTA); 0.5 % SDS; 300 m*M* sodium acetate, pH 6.
9. 10 mg/mL Proteinase K.
10. TE saturated phenol:chloroform (1:1).
11. Isopropanol.
12. 70% ethanol.
13. 10 mg/mL RNase A.
14. 1X TE buffer: 10 m*M* Tris-HCl, pH 7.5 and 1 m*M* EDTA.
15. Ethidium bromide: Prepare as a stock of 10 mg/mL in 1X TE.

16. DNA labeling kit.
17. 0.2 mL thin-wall polymerase chain reaction (PCR) tubes.
18. Taq DNA polymerase and reaction buffer.

# 3. Methods

## *3.1. Transformation of* A. tumefaciens *With Binary Vector (*see also *Chapter 3, Volume 1)*

### *3.1.1. Preparation of Competent Cells*

1. Streak *Agrobacterium* cells stored in 20% glycerol stock at –80°C on LB agar plate amended with appropriate antibiotic (30 µg/mL of rifampicin for strain EHA105 and 100 µg/mL of carbenicillin for strain AGL1).
2. Incubate the plate at 25 to 28°C until colonies appear (about 2 d).
3. Inoculate a single colony into 4 mL LB containing appropriate antibiotic and grow at 28°C, 250 rpm to log phase ($OD_{600}$ = 0.3–0.6).
4. Inoculate the 4 mL culture into 100 mL LB in a 500 mL flask and grow at 28°C, 250 rpm to $OD_{600}$ = 0.5.
5. After chilling the culture for 10 min on ice, pellet *Agrobacterium* cells by centrifugation at 3000*g* for 10 min at 4°C.
6. Remove LB and resuspend the cells with 2 mL of 20 m*M* $CaCl_2$.
7. After dispensing cells into 1.5 ml microcentrifuge tubes (100–200 µL/tube), freeze them in liquid nitrogen and store at –80°C until needed.

### *3.1.2. Transformation (*see *Note 11)*

1. Add 1 µg of vector DNA (in about 5 µL) in 1.5-mL microcentrifuge tube.
2. Thaw competent cells on ice.
3. Dispense 50 µL of competent cells into each microcentrifuge tube containing DNA and mix them by gently pipetting up and down 2 to 3 times.
4. Freeze in liquid nitrogen for 5 min (*see* **Note 12**).
5. Heat shock the frozen cells at 37°C for 20 min.
6. After adding 0.7 mL of LB, grow cells at 28°C at 250 rpm for 2 h.
7. Spread cells on LB agar plate containing 75 µg/mL kanamycin (for all the vectors described in **Subheading 2.4.**) using glass beads (*see* **Note 10**) and incubate the plates (upside down) at 28°C until transformant colonies appear (about 1.5–2 d).
8. Pick two independent transformants and culture them in MM amended with 75 µg/mL kanamycin (*see* **Note 13**).

## *3.2. Transformation of* M. grisea *and* F. oxysporum

### *3.2.1. Preparation of* M. grisea *Spores*

1. Inoculate *M. grisea* on oatmeal agar and grow for 1 to 2 wk under constant fluorescent light at room temperature.
2. Harvest *M. grisea* spores by scraping fungal culture with 1-mL micropipet tip after flooding the plate with 10 mL of sterile water.

3. Filter the spore suspension through two layers of cheesecloth to remove large debris.
4. Determine spore concentration using a hemacytometer. Adjust the spore concentration to $10^6$ spores/mL (*see* **Note 14**). If the original solution is too diluted, concentrate them by resuspending with a smaller volume of water after centrifugation.

#### *3.2.2. Preparation of* F. oxysporum *Spores*

1. Inoculate *F. oxysporum* spores to CMC broth and culture on a rotary shaker (100 rpm) at room temperature for 1 wk (*see* **Note 15**).
2. Filter the culture through two layers of cheesecloth to remove mycelia.
3. Harvest spores by centrifugation at 3000*g* for 5 min followed by 2 washes with sterile water.
4. Resuspend spores in sterile water and adjust the spore concentration to $10^6$ spores/mL (*see* **Note 14**).

#### *3.2.3. Transformation*

1. Incubate the *Agrobacterium* strain containing a binary vector described in **Subheading 2.4.** in 1 mL MM amended with 75 μg/mL kanamycin at 28°C at 250 rpm for 2 d.
2. The *Agrobacterium* cells are diluted to $OD_{600}$ = 0.15 in IM amended with kanamycin and 200 μ*M* acetosyringone (AS) and incubate for an additional 6 h at 28°C at 250 rpm ($OD_{600}$ reaches around 0.6).
3. Mix 100 μL of fungal spore suspension with 100 μL of *Agrobacterium* cells in a microcentrifuge tube and spread the mixture on a nitrocellulose membrane placed on CM in a small Petri plate (15 × 50 mm) (*see* **Note 16**).
4. Incubate the plate for 2 d at 25°C in dark (*see* **Note 17**).
5. Transfer the membrane onto appropriate selection medium amended with hygB for selecting fungal transformants. For *M. grisea*, use complete medium agar amended with 250 μg/mL hygB, 200 μ*M* cefotaxime, and 100 μg/mL moxalactam. *Aspergillus* minimal medium amended with 75 μg/mL hygB, 200 μ*M* cefotaxime, and 100 μg/mL moxalactam is used for selecting *F. oxysporum* transformants (*see* **Note 18**).
6. Incubate the plate at 25°C until hygB-resistant colonies appear (*see* **Note 19**).

### *3.3. Isolation and Purification of Transformants*

1. Prepare 24-well microtiter plates that contain appropriate medium for sporulation: oatmeal agar for *M. grisea*, and CMC broth for *F. oxysporum* (*see* **Note 20**).
2. Transfer a little bit of mycelia from individual transformants to microtiter well using a sterile toothpick or a fine tipped forcep and incubate the plate at 25°C (*see* **Note 21**).
3. For *M. grisea*, flood each well with sterile water and pipet up and down to dislodge conidia. *F. oxysporum* culture in CMC requires no treatment.
4. After plating conidia on solid medium (water agar for *M. grisea* and *Aspergillus* minimal medium for *F. oxysporum*) amended with appropriate antibiotics and incubating for 24 h, pick 1 or 2 single germinating spores for each transformant under microscope and transfer them on appropriate culture medium for subsequent analysis and preservation (*see* **Note 22**).

### 3.4. Molecular Analysis of Transformants

Southern analysis, PCR, or both have been applied for: (1) testing the intactness of a gene (e.g., a reporter gene such as green fluorescent protein [GFP]) introduced via ATMT, (2) determining the copy number of inserted T-DNA, (3) identifying gene knock-out mutants, and (4) isolating genomic regions flanking inserted T-DNA.

#### *3.4.1. Genomic DNA Extraction and Southern Analysis (*see *Note 23)*

1. Prepare 24-well plates containing 1 mL of liquid medium (2YEG for *M. grisea* and potato dextrose broth for *F. oxysporum*). Given the mitotic stability of inserted T-DNA ***(19,20)***, after purifying transformants through single spore isolation, it is not necessary to add antibiotics in growth medium.
2. Inoculate individual transformants to be analyzed in each well and seal the plate with parafilm to prevent evaporation of medium.
3. Grow the transformants for a week at room temperature (RT) on a rotary shaker set at 100 rpm.
4. Grind the cultures in 24-well plate using a hand-made 24-well format grinder (*see* **Note 24**).
5. After adding 0.6 mL extraction buffer and 0.5 µL proteinase K/well, seal the 24-well plate with aluminum foil tape and mix thoroughly by inverting the plate.
6. Incubate at 65°C for 1 h.
7. Transfer the samples into individual microcentrifuge tubes and add 0.6 mL TE saturated phenol:chloroform (1:1). Mix well by inverting the tubes several times.
8. Centrifuge for 10 min at 12,000*g* at RT to separate the two phases.
9. Transfer the aqueous layer to clean tubes and precipitate DNA by adding 420 µL isopropanol (0.7 volume of the sample) and mixing the content.
10. Centrifuge for 10 min at 12,000*g* at RT to pellet DNA.
11. After discarding supernatant, wash DNA with 70% ethanol and dry.
12. Resuspend DNA in 20 to 40 µL TE. Dissolve pellet by gently tapping the tubes.
13. Treat dissolved DNA with 10 mg/mL of 1 µL of RNase A for 30 min at 37°C.
14. Digest 10 µL of DNA with appropriate restriction enzyme for 2 to 3 h in a total volume of 30 µL (*see* **Note 25**).
15. Run digested DNA on 0.7% agarose gel (*see* **Note 26**) and blot the gel on a hybridization membrane.
16. Hybridize the blot with appropriate probe.

#### *3.4.2. Identification of Gene Knock-Out (KO) Mutants by PCR (*see *Note 27)*

1. Design a pair of primers, one corresponding to the 3′- or 5′-end of the positive selection marker (hygB resistance gene) and the other corresponding to a target gene. Design another pair of primers specific to a different gene in transformed fungus for testing the quality of DNA template (*see* **Note 28**).

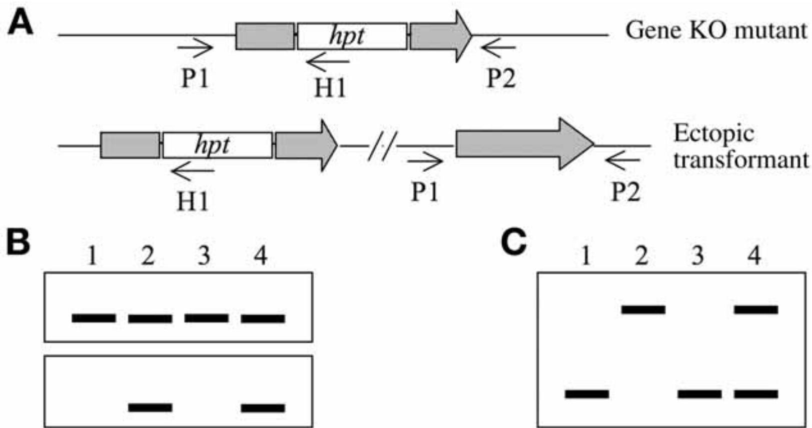


**Fig. 3.** PCR-based screening of gene KO mutants. **(A)** Schematic diagrams of gene KO mutant and ecotopic transformant. Arrows indicate the PCR primers used for PCR screening. Primers P1 and P2 are designed to anneal to the outside of the gene fragment used for creating a mutant allele, and H1 binds to the positive selection marker *hpt*. **(B,C)** Expected PCR product patterns from four different types of fungal strains: lane 1 (wild-type strain), lane 2 (gene KO mutant), lane 3 (ectopic transformant), lane 4 (transformant that has both gene KO and ectopic integration). **(B)** Top panel shows PCR products generated using a pair of primers for testing the quality of PCR template. Bottom panel shows expected PCR products using the primers P1 and H1. **(C)** Expected PCR products based on the primers P1 and P2.

2. Run PCR under appropriate conditions (e.g., primer annealing temperature, extension time according to the expected size of PCR product).
3. Analyze PCR products by running them on agarose gel. Expected patterns are illustrated in **Fig. 3**.

### *3.4.3. Thermal Asymmetric Inter-Laced (TAIL) PCR*

Random, insertional mutagenesis of the fungal genome via ATMT is an efficient forward genetic tool for isolating mutants that are defective in various aspects of fungal biology. Inserted T-DNA provides a molecular tag for isolating genes mutagenized by insertion. TAIL-PCR can be used to isolate the genomic DNA of fungal transformant flanking the site of T-DNA insertion. This method allowed efficient isolation of the host DNA sequence flanking a site of T-DNA insertion in *F. oxysporum* ***(19)***. Plasmid rescue can also be used as an alternative method to TAIL-PCR (*see* **Note 29**).

1. Synthesize primers binding T-DNA border sequence as well as one or more arbitrary degenerate (AD) primer as shown in **Fig. 4** (*see* **Note 30**).
2. Run PCR reactions as previously described ***(19)***.

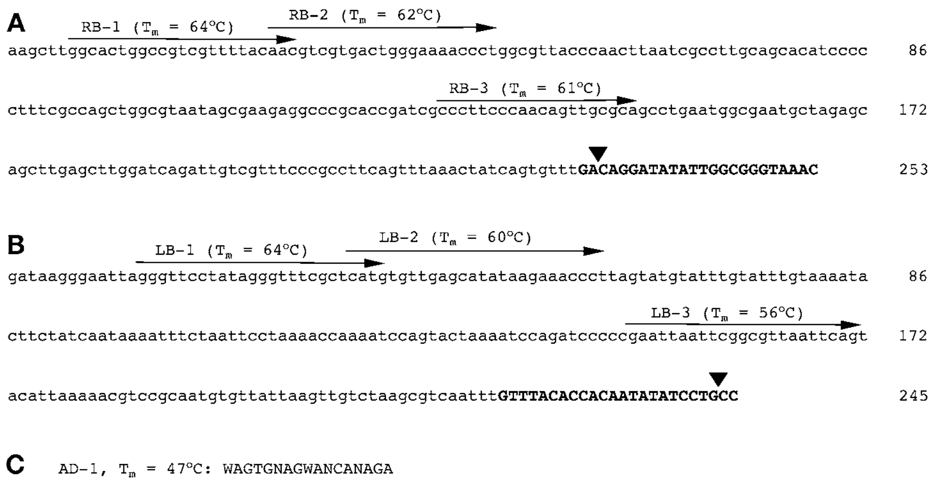


**Fig. 4.** Right **(A)** and left **(B)** border ends of the T-DNA sequence in the vectors described in **Subheading 2.4**. The position and melting temperature ($T_m$) of the primers employed for TAIL-PCR were indicated. Bold uppercase letters indicate the 24-bp imperfect repeat (LB and RB) of the T-DNA, which is cleaved at the indicated positions prior to transfer. The sequence and $T_m$ of the degenerate arbitrary primer (AD-1) is also shown **(C)**. In AD-1, W = A or T, and N = A, C, G, T.

3. Separate PCR products on 1% agarose gel to isolate appropriate fragments (*see* **Note 31**).
4. Sequence the isolated fragments using the outermost specific primer, either RB-3 or LB-3, as the sequencing primer.

### *3.5. Targeted Gene Knock-Out (KO) via ATMT-DS*

1. Transform fungal spores as described above (*see* **Subheading 3.2.**), with *Agrobacterium* cells containing a gene KO vector that carries a mutant allele created by inserting the *hpt* gene flanked by the *tk* gene (a conditional negative selection marker conferring sensitivity to F2dU) under the control of a fungal promoter (*see* **Fig. 1**).
2. HygB-resistant transformants are subjected to negative selection by transferring individual transformants using sterilized toothpicks to a selection medium containing 5 μ*M* F2dU in addition to hygB and cefotaxime (*see* **Note 32**).
3. Incubate the transformants for 3 to 5 d at 25°C in dark.
4. Transfer viable transformants to appropriate medium in 24-well microtiter plates for sporulation.
5. Isolate single spores as described above (*see* **Subheading 3.3.**) and analyze purified transformants for the presence of desired mutation (*see* **Note 33**).

## 4. Notes

1. To ensure phenotypic stability of fungal strains, avoid subculturing them. Repeated subculturing on nutrient-rich medium can potentially lead to loss of virulence and

fertility and reduced conidiation (*29,31*). Thus, it is highly recommended that a fresh culture should be activated from a stock stored at a non-metabolizing state prior to experiment. For long-term storage of *F. oxysporum*, conidia are stored in 15% glycerol at –80°C or fungal cultures grown on carnation leaf agar (*see* **Subheading 2.2.**) are preserved as described in Fisher et al. (*31*) at −20°C. Stock cultures of *M. grisea* are prepared and stored as described by Valent et al. (*29*). After placing sterilized Whatman filter paper disks on oatmeal agar or complete medium plate (*see* **Subheading 2.2.**), fungal culture is allowed to grow over the disks. Once the fungus has completely colonized the disks, the disks are removed and dried. The disks are placed in sterilized post stamp envelope or 2-mL cryo-storage tube and stored at –20°C.

2. Sterilized carnation leaf pieces can be purchased from the *Fusarium* Research Center (FRC) at Penn State (*http://www.cas.psu.edu/Docs/CASDept/Plant/FRC/*; 814-863-0145).
3. It can take several hours for CMC to dissolve completely. While vigorously stirring the solution, add CMC small portion at a time so that it does not form a big clump. Heating shortens the time needed for dissolving CMC.
4. MM (sans $FeSO_4$ and an appropriate antibiotic) can be stored at 4°C for several months. Appropriate amount of 0.01% $FeSO_4$ and an antibiotic are added into MM prior to growing *Agrobacterium* strains.
5. IM (sans $FeSO_4$, MES, an appropriate antibiotic, and AS) can be stored at 4°C for several months. For preparing CM, after autoclaving and cooling down the medium to around 70°C, add 0.01% $FeSO_4$, 1 *M* MES, and acetosyringone as described in Table 1.
6. Prepare antibiotics stock solutions using deionized water and sterilize them by filtering through a 0.22 µm filter. All the antibiotics stock solutions, except hygB (4°C), are stored at −20°C.
7. AS stock solution forms crystals at −20°C. These crystals will quickly dissolve in ethanol when the tube is inverted several times (or briefly vortexted) at room temperature.
8. The filter-sterilized MES stock is stored frozen at −20°C. When the frozen solution is thawed, precipitation appears as white powder. Vortex vigorously until the precipitate has completely dissolved.
9. The vectors described here were derived from pCAMBIA1300 (*http://www.cambia.org/main/r_et_camvec.htm*). Construction scheme for these vectors was previously described (*19,27*).
10. These beads are quite handy for quickly and uniformly spreading bacterial/fungal cells on solid medium, especially when a large number of samples need to be plated (3–4 beads/per 90-mm plate). Wash the beads by soaking them in 1 *N* HCl for several hours. Rinse them with water thoroughly until pH is above 5, dry in a baking oven, and autoclave.
11. Since only one or two *A. tumefaciens* transformants are needed for subsequent step, scaling down the protocol ($^1/_2$ to $^1/_4$) often works fine.
12. Alternatively, freeze cells in –80°C for 15 min.

13. If not used immediately, store these cultures at –80°C until needed as 20% glycerol stock. After fungal transformation, save the one that has yielded higher transformation efficiency as a permanent stock for future use. If one wants to determine whether gene(s) on the introduced binary vector is intact, PCR with primers specific to the target gene can be performed using 1 to 2 μL of intact cells in MM as a template. Alternatively, one can extract plasmid DNA from 5 mL bacterial culture using a commercial silica membrane-based spin column and perform restriction enzyme analysis. Although the yield of plasmid DNA is much lower than that from *E. coli*, we find it sufficient for restriction enzyme analysis.
14. Optimal spore concentration may vary depending on fungal strain/species. The efficiency of transformation via ATMT varied significantly (more than two orders of magnitude) among several fungal species ***(12)***. Even within a species, we noticed significant strain-dependent variation in transformation efficiency ***(19)***. Therefore, determining transformation efficiency for a target strain/species is highly recommended prior to launching a large-scale transformation. The *M. grisea* and *F. oxysporum* strains used here (*see* **Subheading 2.1.**) typically yield 10 and 40 transformants per membrane between (100–400 transformants/1 × $10^6$).
15. Alternatively, culture *F. oxysporum* on carnation leaf agar, which takes much longer than culturing in CMC to produce enough spores for transformation.
16. Use of nitrocellulose membrane does not appear to be essential for successful transformation. The number of plates needed varies depending on the objective of transformation. For generating a pool of insertional mutants randomly tagged by the T-DNA, a large number of plates are needed. However, for introducing a reporter gene (such as the *gfp* gene) to a fungal strain, one plate should be sufficient.
17. Duration of cocultivation is one of the parameters that may require optimization for efficiently transforming a new fungal strain/species. Although transformation efficiency generally increases with the duration of cocultivation in both *F. oxysporum* and *M. grisea* ***(19,20)***, excessive mycelial growth during prolonged co-cultivation might make it difficult to subsequently identify individual transformants. For certain fungal species such as *Fusarium graminearum*, mycelial growth is rapid and excessive during cocultivation, which appears to inhibit bacterial growth and leads to very low transformation efficiency.
18. Geneticin can also be used for selecting transformants from both species (when transformed with a vector carrying the gene conferring resistance to this antibiotic). Optimal hygB (and geneticin) concentration for selecting transformants may vary significantly from one species to another. Sensitivity to these antibiotics can also vary depending on media used. Prior to transforming a new fungal species via ATMT, one must determine the degree of antibiotic sensitivity of the species by inoculating untransformed strain on several media amended with varying concentrations of chosen antibiotic (a 24-well microtiter plate is suitable for this testing). Use a concentration that completely blocks the growth of untransformed strain for selecting transformants.
19. Bacterial cells frequently form a thick lawn on the membrane after 2 d of cocultivation. Reducing the amount of bacterial cells from the membrane seems to

increase the transformation efficiency and speed up the growth of transformants. Place the membrane upside down on selection medium (90-mm Petri plate) for a few minutes. Drag around the membrane on the surface of the medium to remove bacterial cells as much as possible, and subsequently flip and place the membrane on the same medium. Transformant colonies mainly form on the membrane with colonies appearing occasionally outside of the membrane. It takes approx 4 to 7 d to observe transformant colonies.

20. In addition to *hyg*B (or geneticin), add cefotaxime and moxalactam to the medium to ensure that no *Agrobacterium* cells transferred along with fungal transformants can grow.
21. It may take up to 1 wk to produce conidia from *M. grisea*, whereas 2 to 3 d of culture is often sufficient to produce conidia from *F. oxysporum*.
22. Mix a small volume of conidial suspension (1–3 µL) with a sterilized water drop (100–200 µL) on the surface of medium in small Petri plate (50-mm diameter). Spread conidia using glass beads (*see* **Note 10**). For isolating single spores from a large number of transformants, we use the following method: (1) Prepare a thin layer of medium inside the lid of regular Petri plate (90-mm diameter); (2) draw lines on the plastic surface to divide the medium into 16 sections (number them); (3) touch the culture of transformant with the rounded tip of Pasteur pipet (produced by melting the tip in flame); (4) streak spores attached to the tip on the medium as if plating bacterial cells; (5) after sterilizing the pipet tip, plate another transformant to the next section; (6) Cover the medium with the bottom part of Petri plate and seal with parafilm; (7) grow at 25°C for 24 h, and (8) cut out an agar block containing a single germinating conidium using the flattened tip of a pin (or a platinum wire) under a compound microscope (100 × magnification).
23. This DNA extraction protocol is for quickly analyzing transformants and may not be suitable for analyses requiring a high quality genomic DNA preparation. DNA yield from *F. oxysporum* is significantly lower than that from *M. grisea*, mainly as a result of less mycelial growth under this condition. For more genomic DNA, culture *F. oxysporum* transformants in potato dextrose broth in small Petri plates (after inoculation, leave them on lab bench for 1wk).
24. It is not essential to remove the medium. Alternatively, transfer the cultures into individual microcentrifuge tubes after blotting between paper towels and grind them using a Teflon bar. Freeze-drying is not necessary, but doing so increases DNA yield.
25. For determining the copy number of inserted T-DNA, use a restriction enzyme that has no or one recognition site on the T-DNA construct used for transformation. For checking gene disruption, use a restriction enzyme that has no recognition site on the positive selection marker (e.g., hygB-resistance gene) interrupting the target gene.
26. Before running the gel, run 5 µL of digested DNA in 0.7% agarose minigel to confirm complete digestion. If DNA has not been completely digested, further purify genomic DNA. After increasing the volume of DNA solution to 200 µL with TE, repeat a phenol/chloroform extraction. For DNA precipitation, add 10 µL of 4 *M* NaCl and 400 µL of 100% ethanol. Resuspend DNA in TE.

27. PCR analysis can be performed prior to single spore isolation to reduce the number of transformants that need to be purified. A protocol by Xu and Hamer ***(32)*** is suitable for quickly analyzing transformants by PCR without purifying genomic DNA.
28. The target gene-specific primer should anneal to the outside of the gene fragment used for creating a mutant allele (*see* **Fig. 3**) so that when combined with a primer specific to the positive selection marker, only gene KO mutants yield a PCR product. The second pair of primers should produce a PCR product from all transformants and serve to test the quality of PCR template. Alternatively, instead of using two pairs of PCR primers, a single pair of primers that bind to the outside of the mutant allele can be used for both purposes. Ectopic transformants should produce a PCR product that corresponds to the wild-type gene, whereas gene KO transformants produce a PCR product that is larger than that from ectopic transformants due to the presence of the positive selection marker. Although this method requires less PCR reactions than the former method, the size of both PCR products to be amplified is larger than that by the former method, thus requiring a longer PCR extension time. Because it also amplifies two PCR products that differ by the size of the positive selection marker (e.g., 1.4 kb for the *hpt* gene), optimizing PCR conditions might be more difficult.
29. If the border sequences become truncated beyond the annealing site for the amplifying borders, PCR amplification of the desired product would not occur. Although the frequency of T-DNA truncation in *F. oxysporum* and *M. grisea* does not appear to be as high as that in plants, such events have been detected. Unless the truncation is extensive, reaching deeply into the T-DNA, plasmid rescue can solve this problem. The binary vector pKHt (see **Subheading 2.1.**) carries the ColE1 replication of origin and the chloramphenicol resistance gene ***(19)***. Therefore, from mutants generated using pKHt, inserted T-DNA along with its flanking genomic DNA can be cloned into *E. coli* as a replicating plasmid as follows: (1) digest genomic DNA of selected transformant with a restriction enzyme that does not have a recognition site on the T-DNA, (2) ligate digested DNA, and (3) transform *E. coli* and isolate chloramphenicol resistant colonies. The fungal genes tagged by T-DNA of pKHt can also be identified via TAIL-PCR.
30. We designed a 16-nucleotide (nt) long arbitrary degenerate (AD) primer, labeled AD-1, with a calculated melting temperature ($T_m$) of 47°C. $T_m$ of primers specific for each border sequence of the T-DNA (LB-1, 2, and 3 for the left border and RB-1, 2, and 3 for the right border), ranging from 20 to 26 nt in size, were designed to be at least 58°C or higher, as previously recommended ***(33)***. The 5′-end of LB-3 and RB-3 was 74 bp and 63 bp apart from the 5′-end of LB-2 and RB-2, respectively, to facilitate the identification of PCR products corresponding to T-DNA insert junctions by size comparison between the secondary and tertiary reaction products.
31. As a result of the nested arrangement of the RB and LB primers within one another (*see* **Fig. 4**), the size profile of the desired PCR product is staggered, decreasing with each TAIL-PCR reaction. The primary reaction typically produces several products. The number of PCR products is reduced with each successive reaction (*see* **Note 30**). In some transformants, more than one PCR product can be

amplified from a single insertion site, presumably because of the presence of multiple annealing sites of AD-1 in the vicinity of the inserted T-DNA.

32. Ectopic transformants expressing the *tk* gene fail to grow on this medium due to their sensitive to F2dU, whereas gene KO mutants are insensitive to F2dU (*see* **Fig. 1**). Add 100 µL of selection medium in each well of 96-well microtiter plate. Alternatively, prepare the medium in Petri plates and spot transformants in a grid format. The concentration of F2dU required for selecting against ectopic transformants varied significantly from one species to another ***(27)***. The medium used can also affect sensitivity to F2dU. For a new fungal species, determine the degree of its F2dU sensitivity by comparing the growth of *tk* transformants and untransformed strain on several media amended with varying F2dU concentrations (use 24-well microtiter plate for this testing). Use a concentration that completely blocks the growth of transformants expressing *tk*.
33. Certain fraction of transformants after dual selection appears to be false positive (i.e., ectopic transformants insensitive to F2dU) in both *M. grisea* and *F. oxysporum*. This type of transformants mainly results from truncation of the *tk* gene during T-DNA integration ***(27)***. The frequency of false positives varies depending on a number of factors, including fungal species and strains, the target locus, and the length of the gene fragments used for constructing a mutant allele. In fungal species/strains that have a high rate of false positive, PCR prior to single spore isolation (*see* **Note 27**) is highly recommended.

## Acknowledgments

The methods described here were developed using grants from USDA-NRI (2002-02367), the Crop Functional Genomics Center of the 21st Century Frontier Research Program (CG1413), and the Korea Science and Engineering Foundation (Agricultural Plant Stress Research Center). We would like to thank Justin Dillon for critically reviewing the chapter.

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