Chapter 27

Targeted Gene Disruption in Koji Mold *Aspergillus oryzae*

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Abstract

Filamentous fungi have received attentions as hosts for heterologous protein production because of their high secretion capability and eukaryotic post-translational modifications. One of the safest hosts for heterologous protein production is Koji mold *Aspergillus oryzae* since it has been used in the production of Japanese fermented foods for over 1,000 years. The production levels of proteins from higher eukaryotes are much lower than those of homologous (fungal) proteins. Bottlenecks in the heterologous protein production are suggested to be proteolytic degradation of the produced protein in the medium and the secretory pathway. For construction of excellent host strains, many genes causing the bottlenecks should be disrupted rapidly and efficiently. We developed a marker recycling system with the highly efficient gene-targeting background in *A. oryzae*. By employing this technique, we performed multiple gene disruption of the ten protease genes. The decuple protease gene disruptant showed fourfold production level of a heterologous protein compared with the wild-type strain.

Key words: *Aspergillus oryzae*, Filamentous fungi, Multiple gene disruptions, Heterologous protein production, Highly efficient gene-targeting, Marker recycling

1. Introduction

Koji mold *Aspergillus oryzae* is a filamentous fungus that is one of the excellent hosts for heterologous protein production due to its high protein productivity and the safety guaranteed by its use in the manufacture of Japanese fermented foods for over 1,000 years (1). In general, the production level of proteins from animals and plants is much lower than the homologous (fungal) proteins (2–5). In the heterologous protein production of filamentous fungi, proteolytic degradation of the produced protein is one of the bottlenecks limiting the yields (6, 7). For example, *A. oryzae* has 134 protease genes (8), many of which might cause degradation of the heterologous protein. To enhance the ability of protein

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production, it is important to generate a host applicable to multiple rounds of genetic manipulations. However, compared with other microorganisms such as bacteria and yeasts, only a few attempts to manipulate many genes such as multiple disruptions of protease genes had been performed for breeding of industrial strains in filamentous fungi.

In order to carry out multiple gene disruptions rapidly and efficiently, we have developed a marker recycling system with the highly efficient gene-targeting background (9). The *pyrG* gene encoding orotidine-5′-phosphate (OMP) decarboxylase is used for marker recycling, which allows multiple gene disruptions in *A. oryzae*, since *pyrG*-excised strains can be positively selected by using 5-fluoro-orotic acid (5-FOA) that is converted to the toxic intermediate 5-fluoro-UMP by the enzyme (10). In each disruption process, the *pyrG* marker is excised by the direct repeats of ~300 bp upstream flanking region of the target gene, resulting in no residual ectopic/foreign DNA fragments in the genome. For the highly efficient gene-targeting background, *A. oryzae ligD* gene homologous to *Neurospora crassa mus-53* gene involved in nonhomologous chromosomal integration was disrupted, resulting in ~90% gene disruption efficiency that is much higher than the wild type (~40%) (9, 11). By using this system, we could generate a decuple protease gene disruptant showing fourfold higher level production of a heterologous protein than the wild-type strain (11).

2. Materials

2.1. Construction of Gene Disruption Fragments

1. MultiSite Gateway system (Invitrogen, San Diego, CA).
2. PrimeSTAR HS DNA Polymerase (TaKaRa, Otsu, Japan).
3. pDONR™P4-P1R (Invitrogen).
4. pDONR™P2R-P3 (Invitrogen).
5. pgEpG containing the *pyrG* gene (9).
6. pDEST™R4-R3 (Invitrogen).
7. *A. oryzae* RIB40 strain (wild type) (8).

2.2. Transformation of A. oryzae

1. NSPlD1 strain (Δ*ligD* Δ*pyrG* strain) [*niaD*$^{-}$ *sC*$^{-}$ *adeA*$^{-}$ Δ*argB*::*adeA*$^{-}$ Δ*ligD*::*argB* Δ*pyrG*::*adeA*] (9).
2. DPY medium: 2% dextrin, 1% polypeptone, 0.5% yeast extract, 0.5% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, pH 5.5. Mix all together and autoclave.
3. DPY liquid medium containing 20 mM uridine and 0.2% uracil. Uridine and uracil can be added before autoclaving.

4. Sterilized miracloth (Calbiochem, Darmstadt, Germany).
5. TF Solution I: 50 mM maleic acid (pH 5.5), 1% Yatalase (TaKaRa), 0.6 M $(NH_4)_2SO_4$. Prepare immediately before use and ultrafiltrate.
6. TF Solution II: 1.2 M sorbitol, 50 mM $CaCl_2{\cdot}2H_2O$, 35 mM NaCl, 10 mM Tris–HCl (pH 7.5). Mix all together and autoclave.
7. TF Solution III: 60% PEG 4000, 50 mM $CaCl_2{\cdot}2H_2O$, 10 mM Tris–HCl (pH 7.5). Mix all together and autoclave.
8. M+Met medium: 0.2% NH_4Cl, 0.1% $(NH_4)_2SO_4$, 0.05% KCl, 0.05% NaCl, 0.1% KH_2PO_4, 0.05% $MgSO_4{\cdot}7H_2O$, 0.002% $FeSO_4{\cdot}7H_2O$, 2% glucose, 0.15% methionine, pH 5.5. Mix all together and autoclave.
9. Top agar: M+Met medium including l.2 M sorbitol and 0.8% agar. Autoclave.
10. M+Met agar medium containing 1.2 M sorbitol and 1.5% agar. Autoclave.
11. M+Met agar medium containing 1.5% agar. Autoclave.

2.3. Colony PCR for A. oryzae *Transformants*

Colony PCR Master Mix: 2.8 µl sterilized distilled water, 10 µl 2× PCR Buffer for KOD FX, 4 µl 2 mM dNTPs, 0.4 µl 10 pmol/µl primers, 0.4 µl KOD FX (1 U/µl; TOYOBO, Kyoto, Japan).

2.4. Genomic DNA Extraction

1. DPY liquid medium (see item 2 in Subheading 2.2).
2. Liquid nitrogen.
3. Sterilized miracloth (see item 4 in Subheading 2.2).
4. Metal corn (Yasui Kikai, Osaka, Japan): Bullet-shaped metal to break cells.
5. Multi-Beads Shocker (Yasui Kikai).
6. GE Solution: 50 mM EDTA (pH 8.0), 0.5% SDS, 0.1 mg/ml Proteinase K. Prepare immediately before use.
7. Ethanol precipitation solution: 40 ml ethanol, 1.6 ml 3 M sodium acetate (pH 5.2). Prepare immediately before use.
8. RNase TE: 5 ml TE (10 mM Tris–HCl (pH 8.0), 1 mM EDTA (pH 8.0)), 5 µl 20 mg/ml RNase A solution. Prepare immediately before use.
9. PCI: phenol/chloroform/isoamyl alcohol (25:24:1).
10. CI: chloroform/isoamyl alcohol (24:1).
11. 70% ethanol.
12. TE: 10 mM Tris–HCl (pH 8.0), 1 mM EDTA (pH 8.0).

2.5. Southern Analysis

1. Agarose gel electrophoresis equipment and agarose gel.
2. Restriction enzymes and 10× buffers.

3. Hybond N+ membrane (GE Healthcare, Piscataway, NJ).
4. ECL (enhanced chemiluminescence) direct nucleic acid labeling and detection system (GE Healthcare).
5. LAS-100plus luminescent image analyzer (Fuji Photo Film, Tokyo, Japan).

2.6. Positive Selection of pyrG-Excised Strains by Using 5-FOA

1. 1.6 mg/ml 5-FOA solution. Dissolve in distilled water at 55°C with shading and then ultrafiltrate.
2. 1 M uridine solution. Ultrafiltrate.
3. PD agar medium containing 0.8 mg/ml 5-FOA and 20 mM uridine/0.2% uracil. Autoclave 100 ml 2× PD (Potato/dextrose) agar (Nissui Phamaceutical, Tokyo, Japan) containing 0.4% uracil. After cooled, add 100 ml 1.6 mg/ml 5-FOA solution and 4 ml 1 M uridine. Pour into plates and store in dark.

3. Methods

For high gene disruption frequency, we previously generated a disruptant of the *ligD* gene encoding DNA ligase IV homolog involved in the final step of nonhomologous end joining (9) with the selective marker *argB* in the *A. oryzae* quadruple auxotrophic strain, NSAR1 (*niaD*$^{-}$ *sC*$^{-}$ *adeA*$^{-}$ Δ*argB*::*adeA*$^{-}$) (12). The Δ*ligD* strains grow and conidiate comparably to the nondisrupted transformants (Fig. 1a), suggesting that it can be used in experiments such as heterologous protein production. On the other hand, the Δ*ligD* strain reduces the growth in the presence of methyl methanesulfonate (MMS), a chemical mutagen.

In order to add uridine/uracil auxotrophy that is applicable to marker recycling and multiple gene disruption, the *pyrG* gene was disrupted with the selective marker *adeA* in the Δ*ligD* strain (NSR-ΔlD2). The Δ*ligD* Δ*pyrG* strain (NSPlD1) can be transformed with the plasmid harboring the wild-type *pyrG* gene. The transformants are able to grow in the absence of uridine/uracil while the Δ*ligD* Δ*pyrG* strain does not form colonies on the same medium (Fig. 1b). However, only the Δ*ligD* Δ*pyrG* strain shows resistance against 5-FOA. These results demonstrate that positive selection using 5-FOA for uridine/uracil auxotrophs can be applied to *pyrG* marker recycling in *A. oryzae*.

By using 1.3–1.5 kb flanking regions of the target gene, the disruption efficiency is very high (~90%) in the Δ*ligD* background (9, 11). Transformants derived from the Δ*ligD* Δ*pyrG* strain produce a comparable level of heterologous proteins with the relevant wild-type strain. We further reported that decuple protease gene disruption increased heterologous protein yields (11).

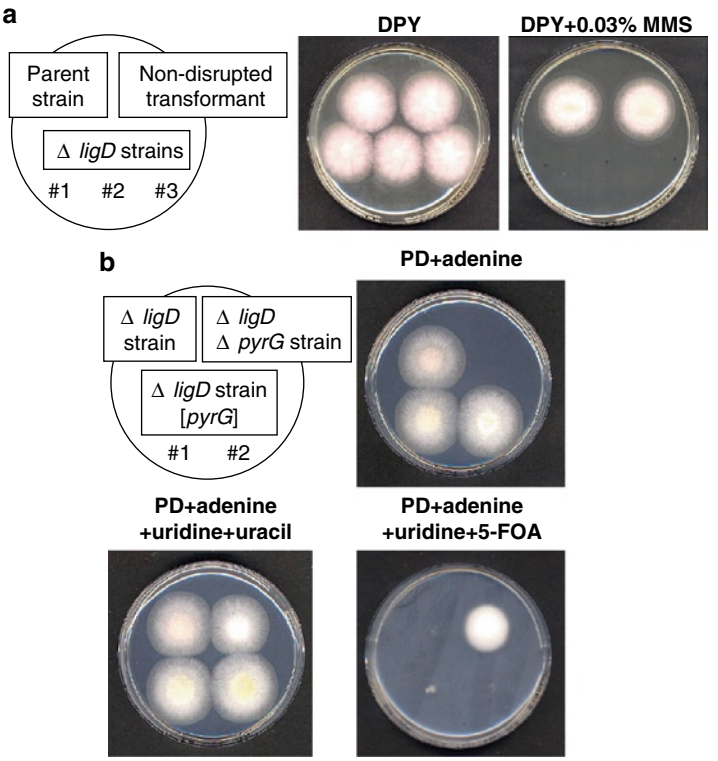

Fig. 1. Growth of the ***A. oryzae* ΔligD ΔpyrG** strain. (**a**) Sensitivity of the **ΔligD** strain to a chemical mutagen MMS. Conidia (~100 conidia/5 μl) were spotted on the DPY agar medium and incubated at 30°C for 3 and 5 days in the absence and presence of MMS, respectively. (**b**) Resistance of the **ΔligD ΔpyrG** strain to 5-FOA. Conidia (~600 conidia/5 μl) were spotted on PD medium with indicated supplements and 5-FOA (0.8 mg/ml). The agar plates were incubated at 30°C for 3 days.

3.1. Construction of DNA Fragments for Gene Disruption in A. oryzae

Plasmid construction for gene disruption fragments is done by the MultiSite Gateway system as instructed by the manufacturer. PCR is performed with the PrimeSTAR HS DNA Polymerase that has a high fidelity.

1. Upstream flanking region (1.3–1.5 kb) of the target gene ORF is amplified with the genomic DNA of *A. oryzae* RIB40 strain as template, and inserted into pDONR™P4-P1R by the BP recombination reaction, generating a 5′ entry clone.
2. The upstream (0.3 kb) and downstream (1.3–1.5 kb) flanking regions of the gene are amplified. The two fragments are connected by fusion PCR (see Note 1), and inserted into pDONR™P2R-P3 by the BP recombination reaction, generating a 3′ entry clone.
3. The obtained 5′ and 3′ entry clones together with a center entry clone plasmid, pgEpG containing the *pyrG* gene (9), are mixed for the LR recombination reaction with the destination

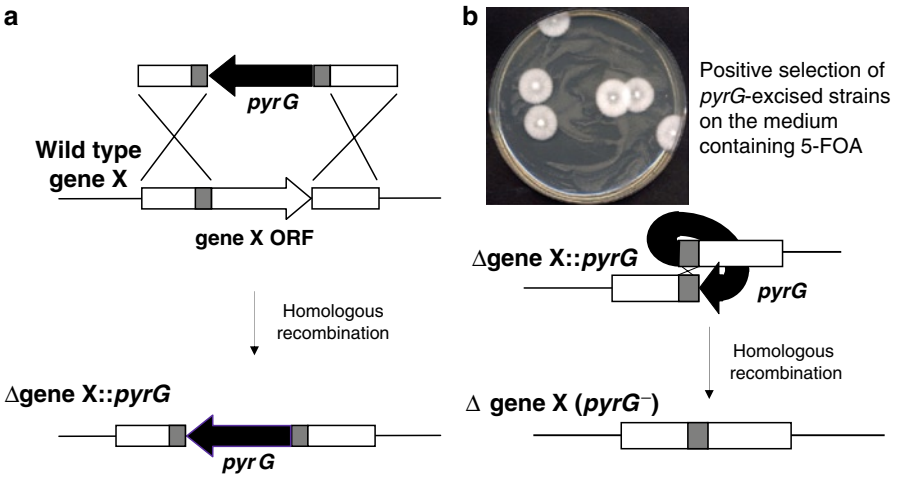

Fig. 2. Overview of multiple gene disruption by *pyrG* marker recycling in *A. oryzae*. (**a**) Targeted gene disruption with the *pyrG* marker. The *boxes* (1.3–1.5 kb) are the flanking regions used for disruption of the target gene. The 0.3-kb upstream flanking region of the target gene (*boxed in gray*) is attached at 5′-end of the downstream flanking regions, introducing direct repeats. (**b**) Excision of the *pyrG* marker targeted at the disrupted locus. By homologous recombination of the direct repeats consisting of the 0.3 kb upstream flanking region of the target gene (*boxed in gray*), the *pyrG* gene targeted at the disrupted locus is excised, and then the upstream and downstream flanking region are directly connected. Note that no ectopic/foreign DNA fragments are left in the genome after excision of the *pyrG* marker.

vector, pDEST™R4-R3, generating a plasmid including the gene disruption fragment.

4. The gene disruption fragment is amplified with the resultant plasmid as template. In this construct, 3′-end of the upstream flanking region of the ORF (~300 bp) is fused with the downstream flanking region of the ORF so that the *pyrG* marker is flanked by the ~300 bp directed repeats (Fig. 2a; gray box).

3.2. Transformation of the A. oryzae ΔligD ΔpyrG *Strain*

This procedure is a modified version according to the method of Kitamoto (1).

1. Inoculate the Δ*ligD* Δ*pyrG* strain in 100 ml DPY liquid medium containing 20 mM uridine and 0.2% uracil. Shake the culture for ~20 h at 30°C.
2. Collect mycelia by filtration with a sterilized miracloth in funnel, and wash them with sterilized distilled water.
3. Incubate the mycelia in 10 ml TF Solution I at 30°C by mild agitation (50 strokes/min) for 3 h. Protoplast formation is checked by microscopic observation.
4. Separate protoplasts from mycelia by filtration through sterilized miracloth. Dilute the protoplast suspension with an equal volume of TF Solution II.

5. Gently precipitate the protoplasts by centrifugation (700 × *g*, 8 min, 4°C; see Note 2) and wash twice with 5–10 ml TF Solution II (see Note 3). Finally, protoplasts are resuspended in TF Solution II with the concentration at 1×10^7–5×10^7/ml.
6. Mix a gene disruption DNA fragment (~3 μg/10 μl; see Note 4) with the protoplast suspension (200 μl) and incubate on ice for 30 min.
7. Mix, in three serial steps, 250, 250, and 850 μl of TF Solution III with the DNA-protoplast mixture and keep at room temperature for 20 min.
8. Dilute the PEG-treated protoplast suspension with 5–10 ml TF Solution II, and centrifuge at 700 × *g* for 8 min at 4°C.
9. Resuspend the protoplasts in 500 μl of TF Solution II.
10. Add aliquots of the protoplast suspension in 4 ml Top agar, and pour the mixture onto M+Met agar medium containing 1.2 M sorbitol that is an osmotic stabilizer.
11. After 3–4 days cultivation at 30°C, transformants are visible on the agar medium. Inoculate them onto new M+Met agar medium (without sorbitol) for a single colony (see Note 5).

3.3. Colony PCR of A. oryzae *Transformants*

1. Design a forward primer annealing to the region immediately upstream of the gene disruption fragment, and a reverse primer annealing to the downstream flanking region of the ORF. Colony PCR using these primers reveals disruption of the target gene and *pyrG* marker excision.
2. Pick up mycelia and conidia from a single colony (see Note 6), and suspend them in 50 μl TE buffer.
3. Add 2 μl of the mycelia/conidial suspension in 18 μl of the Colony PCR Master Mix.
4. Run the PCR program and check the amplification by electrophoresis. Transformants showing disruption of the target gene are taken for Genomic DNA extraction and Southern analysis (see Note 7).

3.4. Genomic DNA Extraction of A. oryzae *Transformants*

1. Transformants are grown in 10 ml DPY liquid medium at 30°C for 16–18 h.
2. Mycelia are harvested by filtration with miracloth or filter paper, and washed with distilled water.
3. Freeze the mycelia (~250 mg in wet weight) in liquid nitrogen together with a metal corn, and break them using Multi-Beads Shocker at 2,000 rpm for 10 s.
4. Freeze them in liquid nitrogen and break the mycelia again.
5. After removing the metal corn, add 600 μl GE Solution and shake gently at 60°C for 30–60 min.

6. Add 700 μl PCI and mix well. Centrifuge at 20,000 × *g* at 4°C for 10 min and take the supernatant (see Note 8).
7. Add 600 μl PCI and mix well. Centrifuge at 20,000 × *g* at 4°C for 5 min and take the supernatant.
8. Add 550 μl CI and mix well. Centrifuge at 20,000 × *g* at 4°C for 5 min and take the supernatant.
9. Add 900 μl ethanol precipitation solution and rotate gently. Centrifuge it at 20,000 × *g* at 4°C for 10 min and remove the supernatant.
10. Dry briefly the pellet and add 400 μl RNase TE. After dissolving, incubate at 37°C for 30 min.
11. Add 400 μl PCI and mix well. Centrifuge at 20,000 × *g* at 4°C for 5 min and take the supernatant.
12. Add 350 μl CI and mix well. Centrifuge at 20,000 × *g* at 4°C for 5 min and take the supernatant.
13. Add 1 ml ethanol precipitation solution and invert several times. Centrifuge at 20,000 × *g* at 4°C for 10 min, and remove the supernatant.
14. Add 1 ml 70% ethanol. Centrifuge at 20,000 × *g* at 4°C for 10 min, and remove the supernatant.
15. Dissolve the pellet with 100 μl TE and store the solution at 4°C (see Note 9).

3.5. Southern Analysis of A. oryzae *Transformants*

1. Digest genomic DNAs with restriction enzymes and load them for agarose electrophoresis.
2. Transfer the digested genomic DNAs onto Hybond N+ membrane.
3. Use ECL direct nucleic acid labeling and detection system and an instrument such as LAS-100plus luminescent image analyzer for labeling and detection.

3.6. Positive Selection of pyrG*-Excised Strains by Using 5-FOA*

This process is performed for positive selection of *pyrG*-excised strains by using agar medium containing 5-FOA (Fig. 2b). The *pyrG* marker inserted at the target locus is excised out by homologous recombination with the direct repeats, in which the flanking regions of the target ORF are directly connected without leaving any ectopic/foreign DNA fragments.

1. Conidia (1×10^6–5×10^6/plate) of the gene disruptants with the *pyrG* marker are spread on PD agar medium containing 0.8 mg/ml 5-FOA and 20 mM uridine/0.2% uracil and then incubated at 30°C.
2. After 4–5 days cultivation, growing colonies are transferred onto another 5-FOA agar medium supplemented with uridine/uracil (see Note 10).

3. The strains are confirmed for *pyrG* marker excision by colony PCR and Southern analysis (see Subheadings 3.3–3.5 and Note 11).

3.7. Successive Round of Gene Disruption and Marker Recycling

Resultant 5-FOA resistant strains are uridine/uracil auxotroph that is therefore applicable to successive rounds of gene disruptions using the *pyrG* marker as instructed above (see Note 12).

4. Notes

1. For fusion PCR, the reverse and forward primers of the upstream (0.3 kb) and downstream flanking regions of the gene should be overlapped.
2. We centrifuge protoplast suspensions without brake.
3. When protoplasts are suspended and mixed, we use wide-mouthed pipettes such as sterile transfer pipettes (Sarstedt, Nümbrecht, Germany).
4. The amount of a gene disruption fragment is enough to obtain ~20 transformants.
5. Since filamentous fungi such as *A. oryzae* are multinuclei, transformants are inoculated onto another selective medium for a single colony to make them homokaryotic. One step of this inoculation process is sufficient to obtain homokaryotic transformants.
6. The region with newly formed conidia on 3-day culture is taken for colony PCR.
7. If colony PCR indicates heterokaryotic (with both bands showing wild type and disrupted gene loci), transformants should be inoculated on another agar plate with selective medium.
8. When genomic DNA solutions are taken, 1,000 μl pipette tips should be used to avoid shearing the genomic DNA.
9. The amount of genomic DNA is enough to be used ~5 times for Southern analysis.
10. With this inoculation condition, six to eight colonies appear per one plate.
11. For genomic DNA extraction, 20 mM uridine and 0.2% uracil are added in DPY medium of the *pyrG*-excised strains.
12. In experiments for heterologous protein production, we transform non-*pyrG*-excised strains with an expression plasmid (11).

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