***Original Article***

DOI: 10.5582/ddt.2025.01084

# Comparing gene-targeting efficiency of *Agrobacterium tumefaciens*-mediated transformation and electroporation in the pathogenic fungus *Trichosporon asahii* JCM2466

**Yasuhiko Matsumoto[1,*], Mei Nakayama[1], Yuta Shimizu[1], Sachi Koganesawa[1], Hiromi Kanai[1], Wakako Hayashi[1], Toma Matsuo[1], Tsuyoshi Yamada[2,3], Takashi Sugita[1]**

[1] Department of Microbiology, Meiji Pharmaceutical University, Tokyo, Japan;
[2] Teikyo University Institute of Medical Mycology, Tokyo, Japan;
[3] Asia International Institute of Infectious Disease Control, Teikyo University, Tokyo, Japan.

**SUMMARY**: *Trichosporon asahii* is a pathogenic fungus that causes severe deep-seated fungal infections in neutropenic patients. Ku70, a key component of the non-homologous end-joining (NHEJ) pathway involved in the repair of DNA double-strand breaks, influences gene-targeting efficiency in *T. asahii* MPU129 strain using electroporation, a gene transfer method. Although phenotypic traits such as morphology and biofilm formation vary among *T. asahii* strains, the impact of different gene transfer methods on gene-targeting efficiency remains poorly characterized. In this study, we compared the gene-targeting efficiency of *Agrobacterium tumefaciens*-mediated transformation (ATMT) and electroporation. In *T. asahii* JCM2466 (CBS2479), a strain with high hyphal-forming ability, the *ku70* gene-deficient mutant exhibited a higher gene-targeting efficiency *via* ATMT than the wild-type strain when generating a *cnb1* gene-deficient mutant. The *cnb1* gene encodes the β-subunit of calcineurin. In contrast, in the *ku70* gene-deficient background of *T. asahii* JCM2466, *cnb1*-deficient mutants could not be generated by electroporation. The gene-targeting efficiencies of ATMT and electroporation in the *ku70* gene-deficient mutant of *T. asahii* JCM2466 were 18% and 0%, respectively. The *cnb1* gene-deficient mutants exhibited sensitivity to high temperature and several stress-inducing compounds. These results suggest that ATMT is a suitable gene transfer method for generating gene-deficient mutants in the *ku70*-deficient *T. asahii* JCM2466 background. Therefore, the choice of gene transfer method should be carefully tailored to the genetic background and phenotypic characteristics of each *T. asahii* strain.

***Keywords***: *Trichosporon asahii*, gene transfer method, *ku70* gene, gene-deficient mutant

## 1. Introduction

*Trichosporon asahii*, a basidiomycete yeast, is widely distributed in a variety of environments including soil and plants (*1,2*). Moreover, *T. asahii* is part of the normal microflora of the human skin, digestive tract, and respiratory system (*3-6*). In immunocompromised individuals, including neutropenic patients, *T. asahii* causes severe deep-seated fungal infections (*7-9*). The mortality rate of deep-seated mycosis caused by *T. asahii* is approximately 80% (*10,11*). *T. asahii* is resistant to echinocandin antifungals, and infectious diseases caused by *T. asahii* often occur in patients treated with micafungin (*12,13*). Moreover, *T. asahii* strains resistant to antifungals such as amphotericin B and fluconazole have been isolated from patients (*14,15*). Therefore, gaining insight into the infection mechanisms and drug-resistant systems of *T. asahii* is crucial. *T. asahii* JCM2466 (type strain CBS2479, Riken BioResource Research Center) is widely used in research (*16,17*). The genome information of *T. asahii* JCM2466 (CBS2479) has been published (*18*). In *T. asahii* MPU129, a clinical isolate that exhibits high virulence in a silkworm infection model, several genetic approaches, including a gene-knockdown method, were developed (*19*).

Repair mechanisms for DNA double-strand breaks affect gene-targeting efficiency by introducing homologous DNA fragments. Homologous recombination (HR) and non-homologous end joining (NHEJ) repair are double-strand break repair mechanisms (*20*). HR is required to introduce mutations into a gene-targeting system using homologous DNA fragments (*20,21*), whereas NHEJ repair mediates the insertion of introduced homologous DNA fragments into genome sites that differ from the target region (*20*). Therefore, NHEJ repair leads to a decrease in the gene-targeting efficiency by HR (*20,22*). Ku70 and Ku80 heterodimers play essential roles in NHEJ repair (*23*). In several fungi, the lack of these proteins leads to increased gene-targeting efficiency for generating gene-deficient mutants (*22,24,25*). In *T. asahii* MPU129, the *ku70* gene is involved in the gene-targeting efficiency (*19*). Therefore, the *ku70* gene-deficient mutant of *T. asahii* JCM2466 may be a useful parental strain for generating gene-deficient mutants by HR.

*Agrobacterium tumefaciens*-mediated gene transformation (ATMT) is an advantageous method for gene recombination in several fungi (*26,27*) and a more efficient method than electroporation for obtaining gene-deficient mutants (*28,29*). In 2020, ATMT was used to generate a transgenic *T. asahii* JCM2466 strain expressing enhanced green fluorescent protein (eGFP) (30). On the other hand, a transgenic *T. asahii* JCM2466 wild-type strain expressing eGFP was not generated by electroporation. In the *ku70* gene-deficient mutant of *T. asahii* MPU129, electroporation was used as a gene transfer method to generate the gene-deficient mutants (*19,31,32*) . However, efficiency to generate gene-deficient mutants in the *ku70* gene-deficient mutant *T. asahii* JCM2466 regarding gene transfer methods was not yet compared.

Calcineurin is a calcium-calmodulin-activated phosphatase consisting of a heterodimer with the catalytic and regulatory subunits Cna1 and Cnb1 (*33*). The calcineurin complex with calmodulin controls the expression of several genes by dephosphorylating the transcriptional regulator Crz1 (*34,35*). The *cnb1* gene-deficient *T. asahii* MPU129 mutants exhibited sensitivities to high temperature, cell membrane stress, cell wall stress, and endoplasmic reticulum (ER) stress (*19*). Moreover, the *cnb1* gene was used to evaluate gene-targeting efficacy in *T. asahii* (*36*).

In the present study, we found that ATMT was more efficient method to generate the *cnb1* gene-deficient mutants than electroporation in the *ku70* gene-deficient mutant of *T. asahii* JCM2466. In the *ku70* gene-deficient mutant of *T. asahii* JCM2466, the *cnb1* gene-deficient mutant was not obtained by electroporation. Our findings suggest that ATMT is an efficient method for generating gene-deficient mutants of *T. asahii* JCM2466. Therefore, we assumed that gene transfer method should be selected appropriately in generating *T. asahii* gene-deficient mutants for each strain.

## 2. Materials and Methods

### 2.1. Reagents

Cefotaxime, sodium dodecyl sulfate (SDS), and dithiothreitol (DTT), were purchased from Wako Pure Chemical Industries (Osaka, Japan). Nourseothricin and G418 were purchased from Jena Bioscience (Dortmund, Germany) and Enzo Life Science, Inc. (Farmingdale, NY, USA), respectively.

### 2.2. Culture of *T. asahii*

The *T. asahii* JCM2466 strain used in this study is a standard type strain available from the Riken BioResource Research Center (*https://web.brc.riken.jp/ja/*). Table 1 provides information on the strains used in this study. The *T. asahii* JCM2466 *ku70* gene-deficient mutant was grown on Sabouraud dextrose agar (SDA) containing G418 (50 µg/mL) and incubated at 27°C for 2 days. The *T. asahii* JCM2466 *cnb1* gene-deficient mutant was grown on SDA containing nourseothricin (100 µg/mL) and incubated at 27°C for 2 days.

### 2.3. Construction of gene-deficient *T. asahii* mutants

The plasmid for gene-deficient *T. asahii* strains was constructed according to a previous report (*19*). To generate the *ku70* gene-deficient mutant and the *cnb1* gene-deficient mutant, pAg1-5'-UTR*ku70*-*nptII*-3'-UTR*ku70* and pAg1-5'-UTR*cnb1*-*NAT1*-3'-UTR*cnb1* were used, respectively (*19*). The pAg1-5'-UTR*ku70*-*nptII*-3'-UTR*ku70* plasmid was introduced into *T. asahii* JCM2466 using the previously described ATMT method (*30*). Gene transfer using electroporation was performed according to a previous report (*36*) . The 5'-UTR (*cnb1*) -*NAT1*-3'-UTR (*cnb1*) fragments were amplified by PCR with the primers shown in Table 2. The *T. asahii* competent cells (40 µL) with the DNA fragments (100 ng) were added to a 0.2-cm gap cuvette (Bio-Rad Laboratories, Inc.) and electroporated (Time constant protocol: 1,800 V, 5 ms) using a Gene Pulser Xcell (Bio-Rad Laboratories, Inc.). The cells were suspended by yeast peptone dextrose containing 0.6 M sorbitol and incubated at 27°C for 3 h. After incubation, the cells were applied to SDA containing nourseothricin (300 µg/mL) and incubated at 27°C for 3 days.

### 2.4. Genotyping PCR

**Table 1. *T. asahii* strains used in this study**

| *T. asahii* strains | Relevant genotype | Background | Reference |
|---|---|---|---|
| JCM2466 (Wild-type) | | | Riken BioResource Research Center |
| Δ*ku70* (Parent strain) | *ku70*::*nptII* | JCM2466 | This study |
| Δ*cnb1* | *ku70*::*nptII*, *cnb1*::*NAT1* | JCM2466 Δ*ku70* | This study |

**Table 2. Primers used in this study**

| Primers | Nucleic acid sequence |
|---|---|
| [Genotyping] | |
| Primers-1 for *ku70* genotyping | |
| F *ku70* gene locus | TCGAGGTCGCGACTTTGTTATTGCCAGGTCCTGA |
| R *ku70* gene locus | AGAGCTGCGATCGTGGGCTGATCCGTCC |
| Primers-2 for *ku70* genotyping | |
| F *ku70* gene ORF | TTTCAGCAACTCCGTCAGATCAGCGCCGAAGACA |
| R *ku70* gene ORF | ATCTGCGAAAGAGCGGCCGGGCC |
| Primers-1 for *cnb1* genotyping | |
| F *cnb1* gene locus | GGAGTGAAGAAGGGCAGAGAGCAACAACAGCGGT |
| R *cnb1* gene locus | CCGTGATCGCATGGGGCGTGCACAAAGTG |
| Primers-2 for *cnb1* genotyping | |
| F *cnb1* gene ORF | CGGCTCGGGTACGGTAGACTTCCAGGAGTTTGTCG |
| R *cnb1* gene ORF | AACAGGTCCTCGAGCGTCATCTGCTTGACGATGT |
| Primers-3 for *cnb1* genotyping | |
| F *cnb1* gene outside | GGACGGCGAGCAGGCGCTCTACATGAGC |
| R *cnb1* gene outside | CTGAGTCCCATCGGCCCTTGCCTTCAAGCTACC |
| [Amplification of *cnb1* cassette for electroporation] | |
| F *cnb1*-cassette | CCGTGATCTGCTGCACGTTCGGGTCCG |
| R *cnb1*-cassette | CTGTTCACCTCTGGCTACGACCCCCTCCTC |

Genotyping PCR was performed according to a previous report (*19,36*). To generate the *ku70* gene-deficient mutant, the transformants were grown on SDA containing G418 (300 µg/mL). To generate the *cnb1* gene-deficient mutant, the transformants were grown on SDA containing nourseothricin (300 µg/mL). Colony PCR for *ku70* genotyping and *cnb1* genotyping was performed using primers (Table 2). The mutation in the genome of the transformants was confirmed using the extracted genome by PCR using the primers shown in Table 2.

### 2.5. Temperature sensitivity test

A temperature sensitivity test was performed according to the previous report (*31*). The *T. asahii* strains were grown on SDA and incubated at 27°C for 2 days. *T. asahii* cells were suspended in a physiologic saline solution (0.9% w/v NaCl) and filtered through a 40-µm cell strainer (Corning Inc., Corning, NY, USA). Absorbance of the *T. asahii* cell suspension at 630 nm was adjusted to 1. A series of 10-fold dilutions of the fungal suspension was prepared using saline. Cell suspensions (5 µL each) were spotted on the SDA. The agar plates were incubated at 27°C, 37°C, or 40°C for 24 h, and photographs were obtained.

For growth on liquid medium, Sabouraud liquid medium (1% hipolypepton, 4% dextrose) was used in this study. Suspensions of the *T. asahii* parent strain (Parent) and three *cnb1* gene-deficient mutants (Δ*cnb1* #1, #2, and #3) were prepared with Sabouraud medium and adjusted to 0.005 absorbance at 630 nm ($A_{630}$). The *T. asahii* cells were incubated at 27°C, 37°C, or 40°C for 4 days. $A_{630}$ value was measured using a microplate reader (iMark™ microplate reader; Bio-Rad Laboratories Inc., Hercules, CA, USA).

### 2.6. Drug sensitivity test

A drug sensitivity test was performed according to the previous report (*31*). The *T. asahii* strains were grown on SDA and incubated at 27°C for 2 days. *T. asahii* cells were suspended in physiologic saline solution (0.9% w/v NaCl) and filtered through a 40-µm cell strainer (Corning Inc.). The $A_{630}$ value of the *T. asahii* cell suspension was adjusted to 1. A series of 10-fold dilutions of the fungal suspension were prepared using saline. Cell suspensions (5 µL each) were spotted on the SDA containing SDS (0.01%), Congo red (300 µg/mL), tunicamycin (1 µg/mL), or DTT (12 mM). Each agar plate was incubated at 37°C for 24 h, and photographs were obtained.

## 3. Results

### 3.1. Generating the *ku70* gene-deficient mutant in *T. asahii* JCM2466 by ATMT

*A. tumefaciens* harboring the targeting plasmid, pAg1-5'-UTR*ku70*-*nptII*-3'-UTR*ku70*, was used to generate a *ku70* gene-deficient mutant of *T. asahii* JCM2466 (Figure 1A). Because pAg1-5'-UTR*ku70*-*nptII*-3'-UTR*ku70* contains the *nptII* gene that leads to resistance against the aminoglycoside G418, the *ku70* gene-deficient *T. asahii* mutant shows G418 resistance by recombination (Figure 1A). Through ATMT, the 192nd colony was selected by colony PCR as a candidate *ku70* gene-deficient mutant

(Figure 1B). The transformant grew on SDA containing G418 (Figure 1C). DNA fragments of the predicted size were amplified by PCR with the genome as a template (Figures 1D and 1E). The results suggest that the *ku70* gene-deficient mutant in *T. asahii* JCM2466 was generated by ATMT.

### 3.2. *ku70* gene deficiency on the growth of *T. asahii* JCM2466

We investigated the effect of *ku70* deficiency in *T. asahii* JCM2466 on the growth. The *ku70* gene-deficient mutant was similar growth to the wild-type at either 27°C, 37°C, or 40°C (Figure 2). These results suggest that the growth of *T. asahii* JCM2466 did not alter by *ku70* gene deficiency.

### 3.3. Gene-targeting efficiency in a *ku70* gene-deficient *T. asahii* mutant for gene transfer methods such as ATMT and electroporation

There are phenotypic differences between *T. asahii*

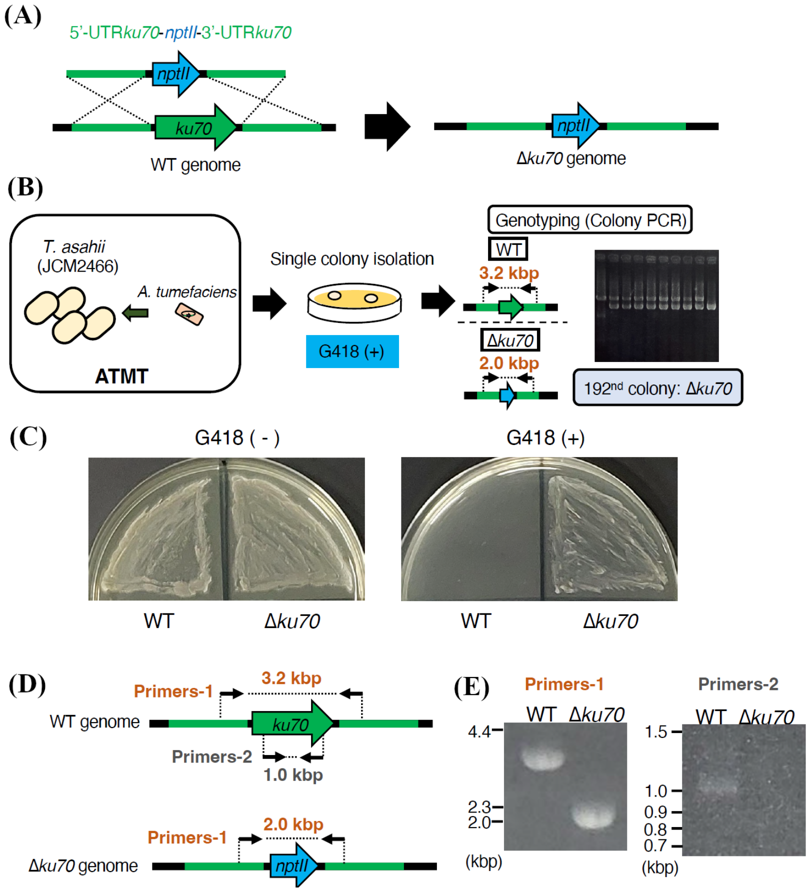

**Figure 1. Establishment of the *ku70* gene-deficient mutant of *T. asahii* JCM2466. (A)** Illustration of replacement of the *ku70* gene in *T. asahii* JCM2466 by recombination. The predicted genome structure of the *ku70* gene-deficient mutant is shown. **(B)** Experimental scheme for obtaining candidate *ku70* gene-deficient mutants of *T. asahii* JCM2466 by the ATMT system. **(C)** The wild-type (WT) and *ku70* gene-deficient (Δ*ku70*) candidate strains were spread on SDA with or without G418 (50 µg/ml) and incubated at 27°C for 2 days. **(D)** Location of the primers for confirming the genome structure of the *ku70* gene-deficient candidate by PCR. **(E)** Confirmation of the *ku70* gene-deficiency of the *ku70* gene-deficient candidate by PCR using extracted genome DNA.

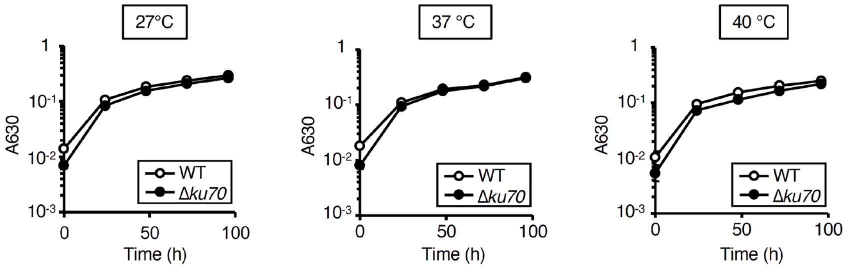

**Figure 2. Growth of the *ku70* gene-deficient mutant in *T. asahii* JCM2466.** The wild-type (WT) and *ku70* gene-deficient mutant (Δ*ku70*) strains were inoculated in Sabouraud liquid medium and incubated at 27°C, 37°C, or 40°C. Absorbance of the culture at 630 nm was monitored.

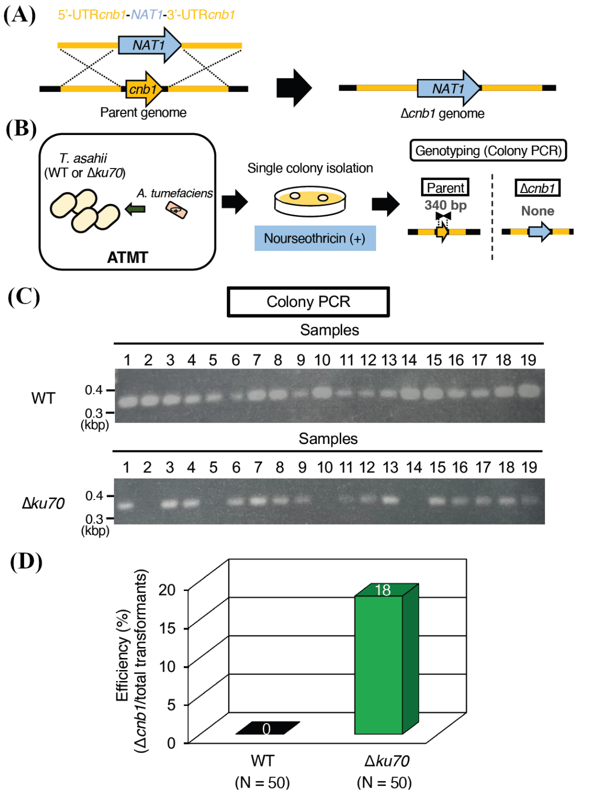

**Figure 3. Increase in the ratio to obtain the gene-deficient mutant of *T. asahii* JCM2466 by *ku70* gene deficiency. (A)** Illustration of replacement of the *cnb1* gene in *T. asahii* JCM2466 by recombination. The predicted genome structure of the *cnb1* gene-deficient mutant is shown. **(B)** Experimental scheme for obtaining candidate *cnb1* gene-deficient *T. asahii* JCM2466 mutants by the ATMT system. **(C)** Colony PCR was performed on colonies obtained from the wild-type (WT) or the *ku70* gene-deficient mutant (Δ*ku70*) grown on SDA containing nourseothricin (300 µg/mL). **(D)** Efficacy of homologous replacement in the *cnb1* gene region. Efficiency (%) was calculated as the ratio of *cnb1* gene-deficient mutants (Δ*cnb1*) per total transformants.

JCM2466 and MPU129. JCM2466 exhibits stronger hyphal formation ability compared with MPU129 (*37*). We examined the usefulness of ATMT for gene targeting in a JCM2466 *ku70* gene-deficient mutant. The gene-targeting efficiency was determined by calculating the ratio of the strain lacking the *cnb1* gene, which encodes the β-subunit of calcineurin (*36*). The *cnb1* gene was deleted by ATMT using pAg1-5'-UTR*cnb1*-*NAT1*-3'-UTR*cnb1* plasmid (Figure 3A). The *cnb1* gene deficiency of nourseothricin-resistant strains was confirmed by colony PCR (Figures 3B and 3C). Of the 50 nourseothricin-resistant colonies obtained from the *ku70* gene-deficient mutant by ATMT, 9 were deficient for the *cnb1* gene (Figure 3D). On the other hand, none of the 50 nourseothricin-resistant colonies obtained from the wild-type by ATMT was deficient for the *cnb1* gene (Figure 3D). In the *T. asahii* MPU129 strain, *ku70* gene deficiency increased the gene-targeting efficiency by electroporation (*19*). Next, we examined the usefulness of electroporation for gene targeting in a JCM2466 *ku70* gene-deficient mutant. Nourseothricin-resistant colonies were obtained from the *T. asahii* JCM2466 and the JCM2466 *ku70* gene-deficient mutant (Figure 4). In all nourseothricin-resistant colonies, the inner region of *cnb1* gene was amplified by colony PCR (Figure 4). These results suggest that ATMT is efficient for gene targeting in a JCM2466 *ku70* gene-deficient mutant compared to electroporation.

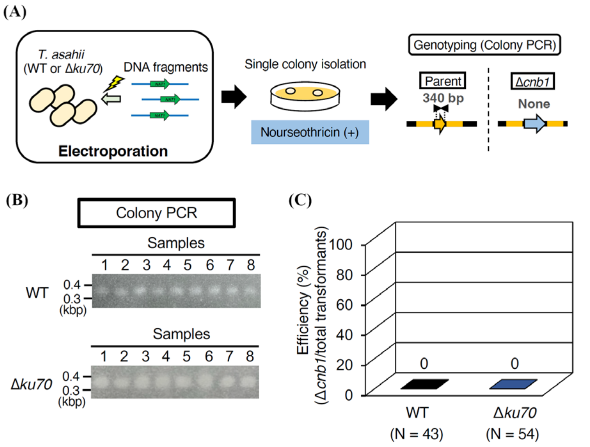

**Figure 4. The ratio to obtain *cnb1* gene-deficient *T. asahii* mutant by electroporation. (A)** Experimental scheme for obtaining candidate *cnb1* gene-deficient *T. asahii* JCM2466 mutants by the electroporation. **(B)** Colony PCR was performed on colonies obtained from the wild-type (WT) or the *ku70* gene-deficient mutant (Δ*ku70*) grown on SDA containing nourseothricin (300 µg/mL). **(C)** Efficacy of homologous replacement in the *cnb1* gene region. Efficiency (%) was calculated as the ratio of *cnb1* gene-deficient mutants (Δ*cnb1*) per total transformants.

### 3.4. Phenotypes of the *cnb1* gene-deficient mutants in *T. asahii* JCM2466

We examined whether *cnb1* gene deficiency affects the stress resistance of *T. asahii* JCM2466. In this experiment, the *ku70* gene-deficient *T. asahii* JCM2466 mutant was used as the parent strain. Moreover, three clones (Δ*cnb1*-#1, #2, and #3), which were selected at random from the candidates of *cnb1* gene-deficient mutants obtained from the *T. asahii* JCM2466 *ku70* gene-deficient mutant by ATMT, were confirmed to have null mutation of the *cnb1* gene by PCR with the primers-1, 2, and 3, and nourseothricin susceptibility assay (Figure 5). Growth of the *cnb1* gene-deficient mutants at 40°C was slower than that of the parent strain (Figure 6). SDS and Congo red damage the cell membrane and cell wall, respectively (*19*). Growth was delayed in the *cnb1* gene-deficient mutants treated with SDS and Congo red (Figure 7). DTT and tunicamycin induce ER stress (*19*). Growth of the *cnb1* gene-deficient mutants was delayed by treatment with DTT and tunicamycin (Figure 7). These results suggest that cellular responses to high temperature, cell membrane damage, cell wall damage, and ER stress, were altered by *cnb1* gene deficiency in the *T. asahii* JCM2466 *ku70* gene-deficient mutant.

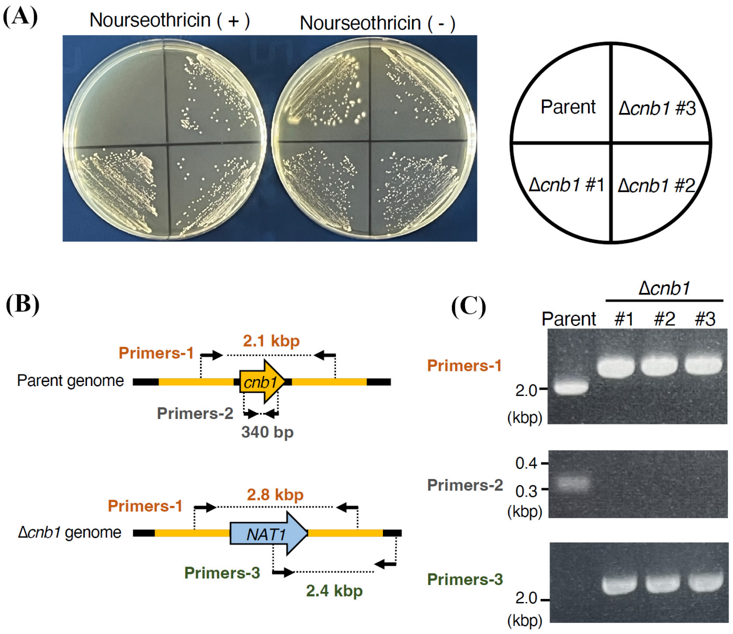

**Figure 5. Confirmation of *cnb1* gene-deficient mutants in *T. asahii* JCM2466. (A)** The *ku70* gene-deficient mutant was used as the parent strain in this experiment. The *T. asahii* parent strain (Parent) and 3 *cnb1* gene-deficient candidates (Δ*cnb1* #1, #2, and #3) were spread on SDA with or without nourseothricin (300 µg/mL) and incubated at 27°C for 2 days. ***(B)*** Location of the primers for confirming the genome structure of the *cnb1* gene-deficient candidates by PCR. **(C)** Confirmation of the *cnb1* gene-deficiency of the *cnb1* gene-deficient candidates by PCR using extracted genome DNA.

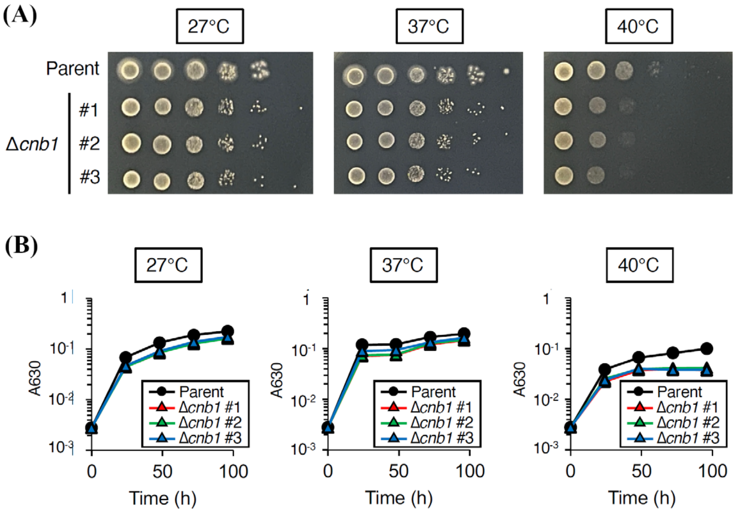

**Figure 6. Temperature sensitivity of the *cnb1* gene-deficient mutants in *T. asahii* JCM2466. (A)** The *ku70* gene-deficient mutant was used as the parent strain in this experiment. The *T. asahii* parent strain (Parent) and 3 *cnb1* gene-deficient mutants (Δ*cnb1* #1, #2, and #3) were grown on SDA and incubated at 27°C for 2 days. *T. asahii* cells were suspended in a physiologic saline solution and filtered through a 40-µm cell strainer. A series of 10-fold dilutions of the fungal suspension were prepared using saline. Cell suspensions (5 µL each) were spotted on the SDA. Agar plates were incubated at 27°C, 37°C, or 40°C for 24 h. **(B)** The *T. asahii* parent strain (Parent) and 3 *cnb1* gene-deficient mutants (Δ*cnb1* #1, #2, and #3) were inoculated in Sabouraud liquid medium and incubated at 27°C, 37°C, or 40°C. The absorbance of the culture at 630 nm was monitored.

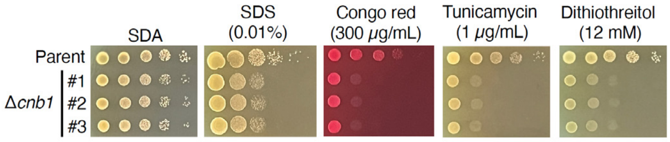

**Figure 7. Sensitivities of *cnb1* gene-deficient *T. asahii* JCM2466 mutants to stress inducers.** The *ku70* gene-deficient mutant was used as the parent strain in this experiment. The *T. asahii* parent strain (Parent) and 3 *cnb1* gene-deficient mutants (Δ*cnb1* #1, #2, and #3) were grown on SDA and incubated at 27°C for 2 days. *T. asahii* cells were suspended in a physiologic saline solution and filtered through a 40-µm cell strainer. A series of 10-fold dilutions of the fungal suspension were prepared using saline. Cell suspensions (5 µL each) were spotted on SDA containing SDS (0.01%), Congo red (300 µg/mL), tunicamycin (1 µg/mL), or DTT (12 mM). Each agar plate was incubated at 37°C for 24 h.

**Table 3. Efficiency of homologous replacement in *cnb1* gene region by electroporation**

| Strain | Total transformants | Homologous replacement (Δ*cnb1*) | Efficiency (%) (Δ*cnb1*/total transformants) |
|---|---|---|---|
| JCM2466 wild type | 51 | 0 | 0% |
| JCM2466 Δ*ku70* | 86 | 0 | 0% |
| MPU129 wild type [a] | 120 | 0 | 0% |
| MPU129 Δ*ku70* [a] | 21 | 4 | 19% |

[a] Data are cited from Matsumoto *et al.* (*19*).

## 4. Discussion

In this study, we obtained a *ku70* gene-deficient mutant of *T. asahii* JCM2466 by ATMT. The *ku70* gene-deficient *T. asahii* JCM2466 mutant generated *cnb1* gene-deficient mutants at a high frequency in ATMT, but not in electroporation. These results suggest that ATMT is an appropriate gene transfer method for generating the gene-deficient mutants in the *ku70* gene-deficient *T. asahii* JCM2466 mutant.

The *ku70* gene-deficient *T. asahii* JCM2466 mutant serves as a valuable parental strain for genetic studies. Deletion of the *ku70* gene did not affect the growth of *T. asahii* JCM2466. Moreover, *ku70* gene deficiency increased gene-targeting efficiency in ATMT. The frequency of obtaining *cnb1* gene-deficient mutants from the *ku70* gene-deficient *T. asahii* JCM2466 mutant by ATMT was 18%. In contrast, *cnb1* gene-deficient mutants could not be obtained from the *ku70* gene-deficient *T. asahii* JCM2466 mutant by electroporation. These finding suggest that the combination of the *ku70* gene-deficient *T. asahii* JCM2466 mutant and ATMT is required for efficient gene targeting. In the *T. asahii* MPU129, however, electroporation proved effective for generating the *cnb1* gene-deficient mutants from the *ku70* gene-deficient mutant (Table 3) (*19*). Therefore, the choice of an appropriate gene transfer method should be tailored to each *T. asahii* strain. The underlying reasons for these strain-dependent differences will be addressed in future studies.

In *Neurospora crassa*, a filamentous fungus belonging to Ascomycota, deletion of the *ku70* or *ku80* genes increases the generating efficiency of gene-deficient mutants to 90-100% (*22*). In *Aspergillus fumigatus*, a filamentous fungus also belonging to Ascomycota, deletion of the *ku80* gene increases the efficiency of obtaining a gene-deficient strain from 3% to 80% (*38*). On the other hand, in *C. neoformans*, which belongs to the same Basidiomycota as *T. asahii*, deletion of the *ku80* gene increased the efficiency of obtaining gene-deficient mutants, but only by 5-6% (*29*). Therefore, HR tends to be less efficient in Basidiomycota than in Ascomycota under the condition of dysfunction of NHEJ repair.

The genome information for *T. asahii* JCM2466 (CBS2479) is published and the correct primers can be ordered; therefore, we assumed that an efficient gene-targeting system in *T. asahii* JCM2466 was established in this study. On the other hand, it is not clear at this time whether targeting efficiencies comparable to that of the *cnb1* locus can be obtained for many other gene loci. Further gene-targeting experiments should be performed in the future. *T. asahii* JCM2466 exhibited a higher hyphal formation ability than MPU129 (*37*). Hyphae possess a larger surface area per cell than yeast. *T. asahii* JCM2466 may offer a greater surface area accessible to *Agrobacterium tumefacies* than MPU129, potentially leading to higher gene transfer efficiency *via* the ATMT system.

Phenotypic analyses of gene-deficient mutants of *T. asahii* JCM2466 can be performed using the gene-targeting method. The *cnb1* gene-deficient mutants exhibited phenotypes such as sensitivities to high temperature, cell membrane stress, cell wall stress, and ER stress inducers. These phenotypic characteristics were also recognized in the same type of mutants which had been generated from *T. asahii* MPU129 (*19,31*). These findings suggest that the *cnb1* gene is responsible for the stress responses of both *T. asahii* JCM2466 and MPU129. Therefore, the combined use of the *ku70* gene-deficient *T. asahii* JCM2466 mutant and ATMT is useful for phenotypic analyses of gene-deficient mutants.

In conclusion, we established an efficient gene-targeting system for standard type strain JCM2466 of *T. asahii* using the *ku70* gene-deficient mutant with ATMT. In electroporation, the gene targeting efficiencies of the *ku70* gene-deficient *T. asahii* JCM2466 mutant and the *ku70* gene-deficient *T. asahii* MPU129 mutant were 0% and 19%, respectively (Table 3). These findings suggest that optimization of the gene transfer method may be necessary to establish an efficient gene-targeting system for each *T. asahii* strain.

## Acknowledgements

We thank Renta Endo, Momoka Yagi, and Haruka Ogino (Meiji Pharmaceutical University) for technical assistance in rearing the silkworms. We also thank SciTechEdit International LLC (Highlands Ranch, CO, USA) for editorial support.

*Funding*: This study was supported by JSPS KAKENHI grant number JP23K06141 (Scientific Research (C) to Y.M.) and in part by the Research Program on Emerging and Re-emerging Infectious Diseases of the Japan Agency for Medical Research and Development, AMED (Grant number JP24fk0108679h0402 to T.S.).

*Conflict of Interest*: The authors have no conflicts of interest to disclose.

## References

1. Sugita T, Nishikawa A, Ichikawa T, Ikeda R, Shinoda T. Isolation of *Trichosporon asahii* from environmental materials. Med Mycol. 2000; 38:27-30.
2. Sugita T, Ichikawa T, Matsukura M, Sueda M, Takashima M, Ikeda R, Nishikawa A, Shinoda T. Genetic diversity and biochemical characteristics of *Trichosporon asahii* isolated from clinical specimens, houses of patients with summer-type-hypersensitivity pneumonitis, and environmental materials. J Clin Microbiol. 2001; 39:2405-2411.
3. Colombo AL, Padovan ACB, Chaves GM. Current knowledge of *Trichosporon* spp. and Trichosporonosis. Clin Microbiol Rev. 2011; 24:682-700.
4. Zhang E, Sugita T, Tsuboi R, Yamazaki T, Makimura K. The opportunistic yeast pathogen *Trichosporon asahii* colonizes the skin of healthy individuals: analysis of 380 healthy individuals by age and gender using a nested polymerase chain reaction assay. Microbiol Immunol. 2011; 55:483-488.
5. Gouba N, Raoult D, Drancourt M. Eukaryote culturomics of the gut reveals new species. PLoS ONE. 2014; 9:e106994.
6. Cho O, Matsukura M, Sugita T. Molecular evidence that the opportunistic fungal pathogen *Trichosporon asahii* is part of the normal fungal microbiota of the human gut based on rRNA genotyping. Int J Infect Dis. 2015; 39:87-88.
7. Walsh TJ, Melcher GP, Lee JW, Pizzo PA. Infections due to *Trichosporon* species: new concepts in mycology, pathogenesis, diagnosis and treatment. Curr Top Med Mycol. 1993; 5:79-113.
8. Júnior JN de A, Hennequin C. Invasive *Trichosporon* Infection: a Systematic Review on a Re-emerging Fungal Pathogen. Front Microbiol. 2016; 7:1629.
9. Duarte-Oliveira C, Rodrigues F, Gonçalves SM, Goldman GH, Carvalho A, Cunha C. The Cell Biology of the *Trichosporon*-Host Interaction. Front Cell Infect Microbiol. 2017; 7:118.
10. Krcmery V, Mateicka F, Kunová A, Spánik S, Gyarfás J, Sycová Z, Trupl J. Hematogenous trichosporonosis in cancer patients: report of 12 cases including 5 during prophylaxis with itraconazol. Support Care Cancer. 1999; 7:39-43.
11. Girmenia C, Pagano L, Martino B, D'Antonio D, Fanci R, Specchia G, Melillo L, Buelli M, Pizzarelli G, Venditti M, Martino P; GIMEMA Infection Program. Invasive infections caused by *Trichosporon* species and *Geotrichum capitatum* in patients with hematological malignancies: a retrospective multicenter study from Italy and review of the literature. J Clin Microbiol. 2005; 43:1818-1828.
12. Kimura M, Araoka H, Yamamoto H, *et al.* Micafungin Breakthrough Fungemia in Patients with Hematological Disorders. Antimicrob Agents Chemother. 2018; 62:324.
13. Kimura M, Asano-Mori Y, Sakoh T, *et al.* Factors associated with breakthrough fungemia caused by *Candida*, *Trichosporon*, or *Fusarium* species when the breakthrough fungemia developed in patients with hematological disorders. Antimicrob Agents Chemother. 2022; 66:e0208121.
14. Toriumi Y, Sugita T, Nakajima M, Matsushima T, Shinoda T. Antifungal pharmacodynamic characteristics of amphotericin B against *Trichosporon asahii*, using time-kill methodology. Microbiol Immunol. 2002; 46:89-93.
15. Iturrieta-González IA, Padovan ACB, Bizerra FC, Hahn RC, Colombo AL. Multiple species of *Trichosporon* produce biofilms highly resistant to triazoles and amphotericin B. PLoS ONE. 2014; 9:e109553.
16. Kurakado S, Miyashita T, Chiba R, Sato C, Matsumoto Y, Sugita T. Role of arthroconidia in biofilm formation by *Trichosporon asahii*. Mycoses. 2020; 185:377-347.
17. Aoki K, Yamamoto K, Ohkuma M, Sugita T, Tanaka N, Takashima M. Hyphal Growth in *Trichosporon asahii* Is Accelerated by the Addition of Magnesium. Microbiol Spectr. 2023; 11:e04242-22.
18. Yang RY, Li HT, Zhu H, Zhou GP, Wang M, Wang L. Draft Genome Sequence of CBS 2479, the Standard Type Strain of *Trichosporon asahii*. Eukaryot Cell. 2012; 11:1415-1416.
19. Matsumoto Y, Nagamachi T, Yoshikawa A, Yamazaki H, Yamasaki Y, Yamada T, Sugita T. Development of an efficient gene-targeting system for elucidating infection mechanisms of the fungal pathogen *Trichosporon asahii*. Sci Rep. 2021; 11:18270.
20. Ding Y, Wang KF, Wang WJ, Ma YR, Shi TQ, Huang H, Ji XJ. Increasing the homologous recombination efficiency of eukaryotic microorganisms for enhanced genome engineering. Appl Microbiol Biotechnol. 2019; 103:4313-4324.
21. Gusa A, Jinks-Robertson S. Mitotic Recombination and Adaptive Genomic Changes in Human Pathogenic Fungi. Genes (Basel). 2019; 10:901.
22. Ninomiya Y, Suzuki K, Ishii C, Inoue H. Highly efficient gene replacements in *Neurospora* strains deficient for nonhomologous end-joining. Proc Natl Acad Sci USA. 2004; 101:12248-12253.
23. Fell VL, Schild-Poulter C. The Ku heterodimer: function in DNA repair and beyond. Mutat Res Rev Mutat Res. 2015; 763:15-29.
24. Yamada T, Makimura K, Hisajima T, Ishihara Y, Umeda Y, Abe S. Enhanced gene replacements in Ku80 disruption mutants of the dermatophyte, *Trichophyton mentagrophytes*. FEMS Microbiol Lett. 2009; 298:208-217.
25. Carvalho NDSP, Arentshorst M, Kwon MJ, Meyer V, Ram AFJ. Expanding the *ku70* toolbox for filamentous fungi: establishment of complementation vectors and recipient strains for advanced gene analyses. Appl Microbiol Biotechnol. 2010; 87:1463-1473.
26. Yamada T, Makimura K, Satoh K, Umeda Y, Ishihara Y, Abe S. *Agrobacterium tumefaciens*-mediated transformation of the dermatophyte, *Trichophyton mentagrophytes*: an efficient tool for gene transfer. Med Mycol. 2009; 47:485-494.
27. Idnurm A, Bailey AM, Cairns TC, Elliott CE, Foster GD, Ianiri G, Jeon J. A silver bullet in a golden age of functional genomics: the impact of *Agrobacterium*-mediated transformation of fungi. Fungal Biol Biotechnol. 2017; 4:6.
28. Chang YC, Kwon-Chung KJ. Complementation of a capsule-deficient mutation of *Cryptococcus neoformans*

restores its virulence. Mol Cell Biol. 1994; 14:4912-4919.

29. Lin X, Chacko N, Wang L, Pavuluri Y. Generation of stable mutants and targeted gene deletion strains in *Cryptococcus neoformans* through electroporation. Med Mycol. 2015; 53:225-234.
30. Matsumoto Y, Yamazaki H, Yamasaki Y, Tateyama Y, Yamada T, Sugita T. A novel silkworm infection model with fluorescence imaging using transgenic *Trichosporon asahii* expressing eGFP. Sci Rep. 2020; 10:10991.
31. Matsumoto Y, Yoshikawa A, Nagamachi T, Sugiyama Y, Yamada T, Sugita T. A critical role of calcineurin in stress responses, hyphal formation, and virulence of the pathogenic fungus *Trichosporon asahii*. Sci Rep. 2022; 12:16126.
32. Matsumoto Y, Sugiyama Y, Nagamachi T, Yoshikawa A, Sugita T. Hog1-mediated stress tolerance in the pathogenic fungus *Trichosporon asahii*. Sci Rep. 2023; 13:13539.
33. Hemenway CS, Heitman J. Calcineurin. Structure, function, and inhibition. Cell Biochem Biophys. 1999; 30:115-151.
34. Kozubowski L, Lee SC, Heitman J. Signalling pathways in the pathogenesis of *Cryptococcus*. Cell Microbiol. 2009; 11:370-380.
35. Yadav V, Heitman J. Calcineurin: The Achilles' heel of fungal pathogens. PLOS Pathog. 2023; 19:e1011445.
36. Matsumoto Y, Nagamachi T, Yoshikawa A, Yamada T, Sugita T. A joint PCR-based gene-targeting method using electroporation in the pathogenic fungus *Trichosporon asahii*. AMB Express. 2022; 12:91.
37. Kurakado S, Miyashita T, Chiba R, Sato C, Matsumoto Y, Sugita T. Role of arthroconidia in biofilm formation by *Trichosporon asahii*. Mycoses. 2021; 64:42-47.
38. Ferreira ME da S, Kress MRVZ, Savoldi M, Goldman MHS, Härtl A, Heinekamp T, Brakhage AA, Goldman GH. The *akuB*(KU80) mutant deficient for nonhomologous end joining is a powerful tool for analyzing pathogenicity in *Aspergillus fumigatus*. Eukaryotic Cell. 2006; 5:207-211.

Received August 13, 2025; Revised October 6, 2025; Accepted October 12, 2025.

**Address correspondence to:*
Yasuhiko Matsumoto, Department of Microbiology, Meiji Pharmaceutical University, 2-522-1, Noshio, Kiyose, Tokyo 204-8588, Japan.
E-mail: ymatsumoto@my-pharm.ac.jp