Targeted gene insertion and replacement in the basidiomycete *Ganoderma lucidum* by inactivation of non-homologous end joining using CRISPR/Cas9

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Abstract

Targeted gene insertion or replacement is a promising genome editing tool for molecular breeding and gene engineering. Although CRISPR/Cas9 works well for gene disruption and deletion in *Ganoderma lucidum*, targeted gene insertion and replacement remains a serious challenge due to the low efficiency of homologous recombination (HR) in these species. In this work, we demonstrate that the DNA double-strand breaks induced by Cas9 were mainly repaired via the non-homologous end joining pathway (NHEJ) at a frequency of 96.7%. To establish an efficient target gene insertion and replacement tool in *Ganoderma*, we first inactivated the NHEJ pathway via disruption of the Ku70 gene (*ku70*) using a dual sgRNA-directed gene deletion method. Disruption of the *ku70* significantly decreased NHEJ activity in *G. lucidum*. Moreover, *ku70* disruption strains exhibited 96.3% and 93.1% frequencies of a targeted gene insertion and replacement when target DNA orotidine 5’-monophosphate decarboxylase gene (*ura3*) with 1.5 kb 5’ and 3’ homologous flanking sequences were used as a donor template, compared to 3.3% and 0% for a control strain (Cas9 strain) at these targeted sites, respectively. Our results indicated that *ku70* disruption strains were efficient recipients for targeted gene insertion and replacement. This tool will advance our understanding of functional genomics in *G. lucidum*.

Keywords: *Ganoderma*, Higher fungi, Medicinal mushroom, Targeted gene insertion, Gene replacement, CRISPR/Cas9
Importance

Functional genomic studies have been hindered in *Ganoderma* by the absence of adequate genome engineering tools. Although CRISPR/Cas9 works well for gene disruption and deletion in *G. lucidum*, targeted gene insertion and replacement has remained a serious challenge due to the low efficiency of homologous recombination in these species, although such precise genome modifications including site mutations, site-specific integrations and allele or promoter replacements would be incredibly valuable. In this work, we inactivated the non-homologous end joining repair mechanism in *G. lucidum* by disrupting the *ku70* using the CRISPR/Cas9 system. Moreover, we established a target gene insertion and replacement method in *ku70*-disrupted *G. lucidum* that possessed high-efficiency gene targeting. This technology will advance our understanding of the functional genomics of *G. lucidum*. 
Introduction

Ganoderma lucidum, a well-known medicinal mushroom, has been used to improve health and treat numerous diseases for over millennium (1). The annual market size of Ganoderma products is more than $2.5 billion USD in Asia (2, 3). Due to its important pharmacological and economic values, it has received world-wide attention in recent years. The complete genome sequence of G. lucidum has recently been published (4, 5), providing a foundation for the further study of Ganoderma biology and biosynthesis of secondary metabolites in this organism. Nevertheless, functional genomic studies have been hindered in Ganoderma by the absence of adequate genome engineering tools.

The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9) system is an efficient tool for genome editing in mammals, plant and microbes (6-10). The feasibility of using CRISPR/Cas9 for generating mutants through short insertion or deletion at targeted DNA double-strand breaks (DSBs) has been also illustrated in mushroom such as Coprinopsis cinerea, Schizophyllum commune and Cordyceps militaris (11-13). Recently, gene disruption and gene deletion have been reported in G. lucidum using the CRISPR/Cas9 system (14-16). To date, however, targeted gene insertion and replacement has remained a serious challenge in Ganoderma, although such precise genome modifications including site mutations, site-specific integrations and allele or promoter replacements would be incredibly valuable.

Targeted gene insertion and replacement can be achieved by a homologous
recombination (HR) using a donor template possessing homology arms. HR-mediated precision repairs in filamentous fungi, however, often occur at a lower frequency than non-homologous end joining (NHEJ)-directed repairs, which is an error-prone repair process (17, 18). Our previous work showed that DNA DSBs directed by CRISPR/Cas9 were predominantly repaired by NHEJ in *G. lucidum* (14). There is competition between NHEJ and HR when DNA DSBs are repaired in eukaryotes such as fungi (19). Therefore, inhibition of NHEJ would be expected to favorably increase the frequency of HR. The HR frequency was shown to be significantly improved by the inhibition of NHEJ in some fungi species such as *S. commune, C. cinerea, Trichoderma reesei*, and *Pleurotus ostreatus* (13, 20-23). However, blocking NHEJ has not been demonstrated yet in *Ganoderma spp.*

In this study, we inactivated the NHEJ repair mechanism in *G. lucidum* by disrupting one of key genes *ku70* using the CRISPR/Cas9 system. Moreover, we established a target gene insertion and replacement method in *ku70*-disrupted *G. lucidum* that possessed high-efficiency gene targeting. This technology will advance our understanding of the functional genomics of *G. lucidum*.

**Results and discussion**

**CRISPR-Cas9-induced DSB repair in *G. lucidum***

We initially tried to insert the *Hgy* cassette into the orotidine 5′-monophosphate decarboxylase gene (*ura3*) by HR in wild-type *G. lucidum*. However, no expected mutants were obtained (data not shown), possibly due to the low frequency of HR in a
wild-type strain. To quantitatively investigate the outcome of CRISPR-Cas9-induced DSB repair, we selected the *G. lucidum* ura3 as a target and transformed the plasmid pU6-ura3-sgRNA1 and the donor pMD19T-ura3-hr-d1 into *G. lucidum* pJW-EXP-intron-opCas9 (Cas9) protoplasts (1 × 10⁷ cells). The strategy for targeted gene (ura3) insertion in *G. lucidum* is shown in Fig. 1A. After selection on the CYM plates with 5-FOA, we obtained a total of 60 FOA-resistant transformants from six independent experiments. Those transformants were confirmed from genomic PCR with the primers ura3-hdu-500-F/ura3-hdd-500-R and Sanger sequencing. The results of the PCR analysis of some transformants are shown in Fig. 1B. Only transformant No. 3 showed a clear 6.9 kb band, but other transformants exhibited different band sizes. Sequence analysis of these PCR products confirmed that CRISPR-Cas9-induced DSBs were repaired by HR in transformant No. 3, and by NHEJ in other transformants. Some sequence results are summarized in supplementary Data S3. Of the 60 transformants selected, two exhibited DSB repair by HR, and 58 showed DSB repair by NHEJ. We observed 3.3% and 96.7% frequencies of HR and NHEJ repair in this study with this strain, respectively (Table 2). These results showed that CRISPR-Cas9-induced DSB induced the frequency of HR in *G. lucidum*, which was similar to reports in plants (24, 25), and NHEJ is the dominant repair mechanism for DSBs in *G. lucidum*.

**Inactivation of the NHEJ pathway in *G. lucidum***

To inactivate the NHEJ pathway in *G. lucidum*, we disrupted the *G. lucidum* ku70 homolog. The *G. lucidum* 5.260125-1 genome reference (4) was analyzed to find a
homolog of the *Coprinopsis cinerea* Ku70 protein using BLASTP. The *gl24233* gene was identified as a candidate *ku70* in *G. lucidum* 5.260125-1. Then, we cloned this putative *ku70* (Supplementary Data 1) from the *G. lucidum* Cas9 strain by PCR using the primers Ku70-F/Ku70-R (Table 1) based on the sequence of the *gl24233* gene. It encodes a protein of 786 amino acids, which showed 60.38, 61.47 and 59.43% identity with the Ku70 proteins from *Coprinopsis cinerea* (GeneBank accession number: KAG2017394.1), *Tricholoma matsutake* 945 (KAF5379661.1), and *Armillaria solidipes* (PBK67889.1), respectively.

To disrupt the *ku70* in *G. lucidum* Cas9, *in vitro*-transcribed *ku70*-sgRNA1, *ku70*-sgRNA2 and plasmid pMD19T-ophph were co-transformed into *G. lucidum* Cas9 strain protoplasts using the dual sgRNA-direct gene deletion method (14). Hygromycin-resistant colonies were isolated and analyzed by PCR using the primers *ku70*-F/ku70-1-R, *ku70*-2-F/ku70-R, and *ku70*-F /ku70-R (Table 1). Sequence analysis of the obtained PCR products using the primers *ku70*-F /ku70-R showed that the region (about 1.5 kb) between the *ku70*-sgRNA1 and *ku70*-sgRNA2 target sites was inverted in transformant No. 5 (Fig. 2). After five rounds of culturing transformant No. 5 in non-selective CYM medium, PCR amplification and sequence analysis were performed to confirm the stability of the *ku70* disruption. The amplified sequences of *ku70* in this *G. lucidum* *ku70* mutant are shown in supplementary data S3. No significant morphology difference was observed between the *G. lucidum* Cas9 strain and *ku70* mutant, although the growth rate of the *ku70* mutant was slightly slower than that of the *G. lucidum* Cas9 strain (Supplementary Fig. S1). Similar
results were also reported in *S. commune*, *Penicillium chrysogenum* and *Botrytis cinerea*, in which the disruption of *ku70* did not significantly affect mycelia growth or morphology (21, 26, 27). The obtained *ku70* mutant was chosen for further analysis.

To evaluate the effect of the *ku70* disruption on NHEJ in *G. lucidum*, the *ura3* gene (Supplementary Data S1) was disrupted in the *G. lucidum* Cas9 strain and *ku70* mutant. The plasmid pU6-*ura3*-sgRNA1 in the absence of a repair template was introduced into protoplasts from the *G. lucidum* Cas9 strain and *ku70* mutant by the PMT method mentioned previously. Transformation of *G. lucidum* Cas9 produced 13, 19, and 17 5′-FOA-resistant colonies on a selective CYM plate in three independent experiments, respectively. Only one 5′-FOA-resistant colony, however, was obtained when the plasmid pU6-*ura3* sgRNA1 was transformed into the *ku70* mutant in the same conditions (Supplementary Data Table S1). The *ura3* gene was amplified from the genome of these transformants and sequenced to determine whether DSBs were repaired by NHEJ. Our results showed that the repair sites of the obtained *G. lucidum* Cas9 transformants were located at 3 bp upstream of the protospacer-adjacent motif (PAM) sequence for the *ura3* sgRNA1, suggesting that these DSBs were repaired by NHEJ. Nevertheless, a 1 bp insertion was found 222-bp downstream of the *ura3* sgRNA1 target site in the *ku70* mutant transformant, which may have been produced by spontaneous mutation. The low frequency of transformation in the Δ*ku70* strain was also been observed in other fungi such as *Aspergillus oryzae*, *S. commune*, and *Stagonospora nodorum* (21, 28, 29). Our results illustrated that NHEJ repair was inactivated in *G. lucidum* by the disruption of the Ku70 function, an essential
component involved in NHEJ repair.

**HR-mediated gene insertion in ku70-deficient G. lucidum**

To evaluate the effects of the inactivation of NHEJ on HR, we transformed the plasmid pU6-ura3-sgRNA1 and the repair template pMD19T-ura3-hr-d1 into the *G. lucidum* Cas9 strain and ku70 mutant, respectively. The obtained 5’-FOA resistant transformants were examined by PCR with the primers ura3-hdu-500-F/ura3-hdd-500-R (Supplementary Data Table S1) and by sequence analysis. Fig. 3 shows that all tested ku70 mutant transformants exhibited a 6.8-kb band, which indicated that this DSB had been repaired by HR-mediated gene insertion. Sequence analysis confirmed target gene insertion in these transformants (Supplementary Data S3). Table 3 shows that the efficiency of HR-mediated gene insertion was roughly 96.3 % (26/27) in the ku70 mutant background, which was 29.2 time higher than that obtained (2/60) in the *G. lucidum* Cas9 background.

**Efficient gene replacement by HR in ku70 mutant**

To investigate whether disruption of ku70 could lead to efficient gene replacement in *G. lucidum*, the plasmids pU6-ura3-sgRNA1 and pU6-ura3-sgRNA2, which targeted two different recognition sities of *ura3*, and the donor template pMD19T-ura3-hr-d2 were co-transformed into the *G. lucidum* Cas9 strain and ku70 mutant. The strategy for targeted gene (*ura3*) replacement for *G. lucidum* is shown in Fig.4. Following 5’-FOA selection, the obtained transformants were subjected to PCR using the primers ura3-hdu-500-F/ura3-hdd-500-R (Table 1) and the resulting PCR products were analyzed by Sanger sequencing. Some of the ku70 mutant transformants (Lane 1,
2, 3, 4, 6, 7) showed 6.8 kb amplicons by PCR, which indicated that the region between the target sites of *ura3*-sgRNA1 and the *ura3*-sgRNA2 were replaced with the *Hyg*’ cassette (Fig. 5A). Sequencing results confirmed gene replacement by HR in these transformants (Supplementary Data S3 and Fig. 5B). However, no corresponding PCR products (6.8 kb) were found in Cas9 transformants (Fig. 5A). Genotyping of the obtained transformants by PCR and sequence analysis showed that 27 of out 29 *ku70* mutant transformants had the *Hyg*’ cassette flanked by homologous regions at the expected locus in three independent experiments, while no corresponding transformants were found out of the 34 Cas9 transformants analyzed (Table 3). Some sequence results are summarized in supplementary Data S3. These results showed that disruption of *ku70* efficiently increased the efficiency of target gene replacement in *G. lucidum*. Previous reports have shown that suppression or disruption of *ku70* leads to increased HR efficiency in fungi (18, 22, 23). The disruption of *ku70* in *Ganoderma* reduced the efficiency of NHEJ leading to HR and increased efficiency of target gene insertion and replacement by HR (30, 31). Gene targeting is important for determining the functions of essential genes and for adding reporters and new markers in studies of gene expression. The obtained *ku70* mutant greatly enhanced gene targeting by HR. Therefore, use the *ku70* mutant will greatly facilitate site-specific integration, site-specific substitution and gene replacement in *G. lucidum*. To eliminate the effect of the introduced plasmid pMD19T-ophph on gene functional analysis, we also constructed a *ku70*-deficiency strain without the plasmid pMD19T-ophph in the genome. In *vitro*-transcribed *ku70*-sgRNA1, *ku70*-sgRNA2
and ura3-sgRNA1 were co-transformed into *G. lucidum* Cas9 strain (Fig. 6). Then, 5’-FOA resistant transformants were isolated and analyzed by PCR using the primers ku70-1-F/ku70-2-R, ku70-1-F/ku70-1-R, and ku70-2-F/ku70-2-R (Fig. 7). Sequence analysis of *ura3* and *ku70* showed that the region between the ku70-sgRNA1 and ku70-sgRNA2 target sites was inverted in transformant No. 1 (Fig. 8). Moreover, 11 bp deletion of *ura3* was detected in the obtained transformant No. 1 (ura3-ku70 mutant). When the repair template pMD19T-ura3-HR (Fig. 9) was transformed into the ura3-ku70 mutant, we obtained the transformant ku70-mutant. The ku70-mutant did not grow on MM plate containing 400 mg/mL 5-FOA, whereas it did grow on MM plate without uridine (Fig. 9). Sequence analysis confirmed that the ku70-mutant was complemented with the *ura3* (Fig 10). Our work will contribute to the progress of the research on this medicinal mushroom.

**Conclusion**

In summary, we showed that the blocking of NHEJ was an efficient way to enhance the efficiency of HR repair of CRISPR/Cas9 induced DSBs in *G. lucidum*. In addition, the constructed *ku70* mutant was suitable for target gene insertion and replacement in *G. lucidum*. This developed method should be helpful in advancing precise genome editing of *Ganoderma* for molecular breeding and biotechnological applications.

**Materials and methods**

**Strains, growth conditions and genomic DNA extraction**
The reference *G. lucidum* pJW-EXP-intron-opCas9 (Cas9) strain used in this study was grown and maintained on potato dextrose agar slants. In *G. lucidum* Cas9 strain, Cas9 was efficiently expressed by codon optimization and addition of the *gpd* intron 1 at 5’ upstream of the opCas9 gene (14). *Escherichia coli* DH5α (Promega, Madison, WI, USA) was used for cloning procedures according to the manufacturer’s instructions. *G. lucidum* transformants were then grown on CYM regeneration plates (0.6 M mannitol, 20 g/l glucose, 10 g/l maltose, 2 g/l yeast extract, 2 g/l tryptone, 4.6 g/l KH₂PO₄, 0.5 g/l MgSO₄ and 10 g/l agar). Genomic DNA was extracted from *G. lucidum* mycelia using the CTAB (cetyltrimethylammonium bromide) method described previously (32).

**Cloning of *G. lucidum* ku70 and in vitro transcription of two ku70 sgRNA cassettes**

The *ku70* of *G. lucidum* was first amplified by genome PCR using the primers *ku70*-F-F and *ku70*-F-R (Table 1). The PCR products were cloned into the pMD19-T vector and confirmed by Sanger sequencing. sgRNAs against these target genes were designed using the web-tool CRISPOR (http://crispor.tefor.net/). The T7 promoter and two *ku70* sgRNA cassettes, T7-*ku70*-sgRNA1 and T7-*ku70*-sgRNA2 (Supplementary Data S2), were synthesized by Sangon Ltd., Corp. (Shanghai, China). These *ku70* sgRNAs were transcribed *in vitro* with a HiScribe™ T7 High Yield RNA Synthesis Kit (NEB, Beijing, China) and purified with a RNA Clean & Concentrator™-25 Kit (Zymo Research, Beijing, China) according to the manufacturer’s protocols.

**Plasmid construction**
The sequence of an intron-codon optimized hygromycin resistance gene (ophph) flanked by NheI and SmaI sites, (Supplementary data S2) was synthesized by Sangon Ltd., Crop (Shanghai, China). This NheI-intron-ophph-SmaI fragment was ligated into the NheI/SmaI site of the plasmid pJW-EXP (33, 34) to produce the pJW-EXP-ophph plasmid. The Hyg<sup>+</sup> cassette, including the glyceraldehyde-3-phosphate dehydrogenase gene (gpd) promoter of G. lucidum, a codon-optimized hph, and the iron-sulfur protein subunit of the succinate dehydrogenase gene (sdhB) terminator, was amplified from the pJW-EXP-ophph using the primers gpd-F and ter-R (Table 1). The pMD19T-ophph plasmid was made by ligating the Hyg<sup>+</sup> cassette into the pMD19-T plasmid (Takara, Dalian, China).

The sequences of pU6-ura3 sgRNA1 and pU6-ura3 sgRNA2 (Supplementary Data S2) were synthesized and ligated into the plasmid pUC57 (http://www.addgene.org/vector-database/4509/) by Shanghai Sangon Ltd., Corp. (Shanghai, China) to produce sgRNA expression plasmids pU6-ura3-sgRNA1 and pU6-ura3-sgRNA2, respectively.

The plasmids pMD19T-ura3-hr-d1 and pMD19T-ura3-hr-d2, which provide donor repair templates for DSBs, were constructed as follows. The flanking sequences (1.5 kb 5′ and 3′) of the ura3 sgRNA1 target site were amplified from genomic DNA using the primers ura3-hru-1-F and ura3-hru-1-R, and ura3-hrd-1-F and ura3-hrd-1-R (Table 1). The ophph cassette was amplified from pJW-EXP-ophph using the primers gpd-F and ter-R (Table 1). The resulting 5′ flanking sequence of the ura3 sgRNA1 target site, the ophph cassette, and the 3′ flanking sequence of the ura3 sgRNA1 target
site were fused into the pMD19T plasmid using the ClonExpress MultiS One Step Cloning Kit (Vazyme, Nanjing, China) to produce the plasmid pMD19T-ura3-hr-d1. The plasmid pMD19T-ura3-hr-d2 was similar to pMD19T-ura3-hr-d1, except that the 3’ flanking sequence of the ura3 sgRNA1 target site was replaced with the 3’ flanking sequence of the ura3 sgRNA2 target site, which was amplified from genomic DNA using primers ura3-hrd-2-F and ura3-hrd-2-R, respectively (Table 1).

**Genetic transformation and screening of transformants**

Plasmid or sgRNA was introduced into *G. lucidum* protoplasts (1×10^7) using the polyethylene glycol-mediated transformation (PMT) method described previously (35, 36). For co-transformation experiments, 10 µg of plasmids and in vitro-transcribed sgRNA were used in this study. ΔKu70 transformants were selected on CYM plates containing 250 mg/L hygromycin B. Transformants were selected for gene insertion or gene replacement on CYM plates containing 400 mg/l 5-fluoroorotic acid (FOA) and 100 mg/l uridine, respectively (Sangon, Shanghai, China).

**Identification of mutant strains**

For identification of the *ku70* disruption transformants, PCR amplification was performed from transformant genomic DNA with the primers Ku70-F/Ku70-1-R (located outside the *ku70*-sgRNA1 target), ku70-2-F/ku70-R (located outside the *ku70*-sgRNA2 target site), and ku70-F/F/ku70-F-R (Table 1). The PCR products from using primers ku70-F/F/ku70-F-R were sequenced directly by Sanger Ltd., Corp. (Shanghai, China).

To identify NHEJ or HR mutations, transformant genomic DNAs were extracted and
amplified for ura3 using the primers ura3-F/ura3-R or ura3-hdu-500-F/ura3-hdd-500-R (Table 1), which were located about 500 bp outside the homology arms. The amplified products were sequenced by the Sanger method to confirm the mutations.

**Data Availability**

The sequence of *G. lucidum ku70* was deposited into *Ganoderma* Genome Data Bank (http://www.herbalgenomics.org/galu/) (4) under the accession number gl24233.

**Acknowledgments**

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**References**


Table 1. Oligonucleotides used in this study

<table>
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<tr>
<th>Target Description</th>
<th>Primers</th>
<th>Sequence (5’ → 3’)</th>
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</thead>
<tbody>
<tr>
<td>The <em>ku70</em></td>
<td><em>ku70</em>-F-F</td>
<td>ATGGCAACCCTATGATGACTGGA</td>
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<td></td>
<td><em>ku70</em>-F-R</td>
<td>TCAGGCCGTGTTGTCAGCG</td>
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<tr>
<td>The ophph cassette</td>
<td>gpd-F</td>
<td>TCCAAAGCGCCTCTCATGG</td>
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<td></td>
<td>ter-R</td>
<td>TGCTCTATGTCCTCTTGTCAG</td>
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<tr>
<td>The 5’ flanking region of <em>ura3</em></td>
<td><em>ura3</em>-hru-1-F</td>
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<td><em>sgRNA1</em> site</td>
<td><em>ura3</em>-hru-1-R</td>
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<td>The 3’ flanking region of <em>ura3</em></td>
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<td><em>sgRNA1</em> site</td>
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<td>The 3’ flanking region of <em>ura3</em></td>
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<td><em>ura3</em>-R</td>
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<td><em>ura3</em>-HR-R</td>
<td>GCGGTAGGATGTCCCTAGGA</td>
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Table 2. Efficiency of NHEJ and HR repair of CRISPR/Cas9 induced DSBs in *G. lucidum*.

<table>
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<tr>
<th>DSB repair</th>
<th>Mutant of <em>ura3</em></th>
<th>Numbers of mutants</th>
<th>Efficiency (%)</th>
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<tr>
<td>NHEJ</td>
<td>Insertion or deletion</td>
<td>58</td>
<td>96.7%</td>
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<td>HR</td>
<td>Target insertion</td>
<td>2</td>
<td>3.3%</td>
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Table 3. Efficiency of targeted gene insertion and replacement in *G. lucidum* Cas9 strain and *ku70* mutant.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Mutant of <em>ura3</em></th>
<th>Exp. 1</th>
<th>Exp. 2</th>
<th>Exp. 3</th>
<th>Efficiency (%)</th>
</tr>
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<tbody>
<tr>
<td>Cas9</td>
<td>Insertion</td>
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<td>1/23</td>
<td>0/17</td>
<td>3.3%</td>
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<tr>
<td><em>ku70</em> mutant</td>
<td>10/10</td>
<td>8/8</td>
<td>8/9</td>
<td></td>
<td>96.3%</td>
</tr>
<tr>
<td>Cas9</td>
<td>Replacement</td>
<td>0/13</td>
<td>0/10</td>
<td>0/11</td>
<td>0%</td>
</tr>
<tr>
<td><em>ku70</em> mutant</td>
<td>7/8</td>
<td>9/9</td>
<td>11/12</td>
<td></td>
<td>93.1%</td>
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</table>

Targeted gene insertions were screened for in 2 out of 60 5’-FOA resistant *G. lucidum* Cas9 transformants produced and in 26 out of 27 5’-FOA resistant *ku70* mutant transformants produced in three independent experiments. Targeted gene replacement was identified for in 0 out of 34 5’-FOA resistant *G. lucidum* Cas9 transformants produced and in 27 out of 29 5’-FOA resistant *ku70* mutant transformants produced in three independent experiments. Exp. Experiment.
Figure legends

Fig. 1. A. Strategy for targeted gene (ura3) insertion in the *G. lucidum* Cas9 strain. B. PCR screening of transformants that underwent homologous recombination using the primers ura3-hru-500-F and ura3-hrd-500-R. The hygromycin resistance cassette *(Hyg')* fused to 1.5 kb of 5' and 3' DNA flanking the *ura3* gene in the donor plasmid pMD19T-uras-hr-d1 and pU6-ura3-sgRNA1 were used for transformation. Arrow indicates the expected 6.8 kb band in transformant No.3.

Fig. 2. A. Strategy for *ku70* disruption in the *G. lucidum* Cas9 strain. *In vitro* transcribed sgRNA1 and sgRNA2 targeting *ku70* and the plasmid pMD19T-ophph were delivered to *G. lucidum* Cas9 protoplasts. B. Sequence analysis of the *ku70* fragment in the *ku70* mutant.

Fig. 3. PCR screening of transformants that underwent targeted gene (ura3) insertion using the primers ura3-hru-500-F and ura3-hrd-500-R in the *ku70* mutant.

Fig. 4. Strategy for targeted gene (ura3) replacement in *G. lucidum*. The *Hyg’* cassette fused to 1.5 kb of 5' and 3' DNA flanking *ura3* (donor pMD19T-uras-hr-d2), pU6-ura3-sgRNA1 and pU6-ura3-sgRNA2 were used for transformation.

Fig. 5. A. PCR screening for transformants that underwent targeted gene (ura3) replacement in *G. lucidum* Cas9 strain and *ku70* mutant. B. Sequence analysis of the targeted gene (ura3) replacement in *ku70* mutant. The primers ura3-hru-500-F and ura3-hrd-500-R were used for PCR amplification.

Fig. 6. Strategy for *ku70* and *ura3* disruption in the *G. lucidum* Cas9 strain.

Fig. 7. A. Selection of 5-FOA-resistant mutants after *ku70*-sgRNA1, *ku70*-sgRNA2
and ura3-sgRNA1 were co-transformed into *G. lucidum* Cas9 strain. Determination of the ura3-ku70 mutant by PCR using primers ku70-1-F/ku70-2-R (B), ku70-1-F/ku70-1-R (C), and ku70-2-F/ku70-2-R (D).

Fig. 8. Sequence analysis of the ura3 (A) and ku70 (B) in WT and ura3-ku70 mutant.

Fig. 9. A. The donor plasmid pMD19T-uras3-HR used for transformation of the ura3-ku70 mutant. B. Subculture of the obtained ku70 mutant, WT and ura3-deficiency strain on MM plate containing 400 mg/mL 5-FOA. C. Subculture of the obtained ku70 mutant, WT and ura3-deficiency strain on MM plate without uridine.

Fig. 10. Sequence analysis of the ura3 in WT and the obtained ku70 mutant.
**A**

<table>
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<th>1</th>
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<th>3</th>
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<th>7</th>
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**Cas9**

- 7 kb
- 4 kb

**ku70 mutant**

- 6.8 kb
- 7 kb

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**B**

- **Cas9**: CCTTGGCAGGCGGCTCTGCTCCG......CAACCGGCTCATACACGGAAAG
- **ku70 mutant**: CCTTGGTCCAAGCGCG...Hyg'...ACATAGAGCA CATACACGGAAAGGAGCC

---

**ku70 mutant**

- C
- T
- C
- G
- T
- G
- C
- A
- A
- A
- G
- C

---

**ku70 mutant**

- A
- C
- A
- T
- A
- G
- A
- G
- C
- A
- C
- A
- C
- G
- G
- A
- A
- G
- A
- G
- C
- C
**ura3-ku70 mutant:**

**A**

**B**

**C**

**D**

- [Image of agar plate with colonies labeled M, WT, 1, 2]
- [Image of gel electrophoresis with markers 5000, 3000, 2000, 1500, 1000]
A

WT: GTCGGTCTGCC CTTTGGCAGGGGGCTTCTGCTCC TCGCCGA

ura3-ku70 mutant: GTCGGTCTTTCTGCTCC TCGCCGA

B

WT: AAGCCCGAGT GACGGTAGGAGGCCCATTCCATCA---Forward ku70---ATGGCCAGTC ACGGGCTCATAGATCCCTTC
AAGCCCGAGT GACTGGCAGGAGGA---Reverse ku70---AATGGCTCCACGGTC ACGGGCTCATAGATCCCTTC

ura3-ku70 mutant:
WT:  

\textbf{\textit{ku70 mutant:}}

\textbf{GTCGGTCTGCC \textcolor{red}{CCT}TGGCAGGGGGCTTCTGCTCC TCGCCGA}

\textbf{GTCGGTCTGCC \textcolor{red}{CCT}TGGCAGGGGGCTTCTGCTCC TCGCCGA}