**RESEARCH NOTE**

**Construction of a CRISPR/Cas9-Mediated Genome Editing System in *Lentinula edodes***

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**ABSTRACT**

CRISPR/Cas9 genome editing systems have been established in a broad range of eukaryotic species. Herein, we report the first method for genetic engineering in pyogo (shiiitake) mushrooms (*Lentinula edodes*) using CRISPR/Cas9. For *in vivo* expression of guide RNAs (gRNAs) targeting the mating-type gene HD1 (*LeA1*), we identified an endogenous *LeU6* promoter in the *L. edodes* genome. We constructed a plasmid containing the *LeU6* and glyceraldehyde-3-phosphate dehydrogenase (*LeGPD*) promoters to express the Cas9 protein. Among the eight gRNAs we tested, three successfully disrupted the *LeA1* locus. Although the CRISPR-Cas9-induced alleles did not affect mating with compatible monokaryotic strains, disruption of the transcription levels of the downstream genes of *LeHD1* and *LeHD2* was detected. Based on this result, we present the first report of a simple and powerful genetic manipulation tool using the CRISPR/Cas9 toolbox for the scientifically and industrially important edible mushroom, *L. edodes*.

*Lentinula edodes* is one of the most widely cultivated edible mushrooms in the world and has great value as a food, seasoning, and pharmacological ingredient. In addition, it has shown promise as a potential cell factory capable of producing a variety of valuable enzymes and organic substances [1,2]. Over the course of domestication of *L. edodes*, several genetic traits including high yield, faster growth rate, color, and specific temperature optima for fruiting body formation have been selected [3]. Despite its importance in the food and industrial markets, proper tools for genetic manipulation, such as genome editing, have not been developed in this important mushroom for use in the research and industrial fields.

The CRISPR/Cas9 system, one of the most powerful and revolutionary genome editing tools, has been used to precisely manipulate the genome of various organisms [4]. In this genome editing system, 20-bp of gRNA recognizes a target sequence that follows a 5'-NGG motif, called a protospacer-adjacent motif (PAM), and Cas9 endonuclease induces a double-strand break (DSB) 3-4 nucleotides upstream of the PAM. This genomic DNA cleavage event is usually repaired by either the nonhomologous end-joining (NHEJ) or homologous recombination (HR) DNA repair system. NHEJ allows for NHEJ-mediated gene disruption with base-pair insertions or deletions, which often results in mutations at the target site due to errors during the repair process. The HR system introduces precise genome editing with appropriate donor DNA. This methodology has recently been successfully used in numerous eukaryotic organisms [5-7]. However, relative to animals or plants, only anecdotal cases of genome editing by CRISPR have been reported in mushroom-forming fungi, including *Coprinopsis cinerea* [8], *Cordyceps militaris* [9], *Schizophyllum commune* [10], *Ganoderma lucidum* [11], *Pleurotus eryngii* [12], and *Pleurotus ostreatus* [13]. The limited availability of genome editing techniques for useful mushroom species has been an obstacle that has prevented advances in the field of genetic and molecular breeding research using fungi.

In this study, we successfully developed and used the CRISPR/Cas9 genome editing system in *L. edodes*. By identifying the appropriate *U6* and active *GPD* promoters from the *L. edodes* genome, we constructed a generic CRISPR/Cas9 plasmid toolkit. Using a PEG-mediated protoplast transformation method, we confirmed that the genome editing
toolbox successfully induces target genome editing in this mushroom.

To prepare proper fungal mycelial materials, the L. edodes cultivar strain Sanjo705 was provided by the Forest Mushroom Research Center (https://www.fmrc.or.kr/). Monokaryotic strains (referred to as Sanjo705-3 and Sanjo705-13) isolated from the spores of Sanjo705 were used as the recipient strains for fungal transformation of CRISPR/Cas9 constructs and further mating assays. The vegetative mycelia of L. edodes were cultured on potato dextrose agar (PDA) and grown at 25 °C in the absence of light.

For identification of a U6 gene promoter-like sequence and subsequent gRNA design, Homo sapiens RN6U-1 (GenBank X07425.1) was used as the query to search for homologs in the L. edodes genome database. A 98.2% identical match was found and named LeU6 snRNA. A 500-bp sequence upstream of the pLeU6 mRNA coding region was aligned with the promoter sequences of H. sapiens RN6U-1 (hRN6U-1). Sequence comparison between hRN6U-1 and LeU6s was carried out by the T-Coffee multiple alignment algorithm in SnapGene® software. To mutate HD1, guide RNAs were designed based on their location in the gene and their GC content. Eight pairs of gRNA spacer sequences are listed in Table 1.

For plasmid construction, cloning of desirable DNA fragments into the vector backbone was performed using T4 DNA ligase and standard ligation protocols (Enzymonics, Daejeon, Korea). The backbone plasmid, pHAtC, was acquired from Addgene (plasmid # 78098). The promoter sequences for pHAtC, was acquired from Addgene (plasmid # 78098). The promoter sequences for fungal transformation of CRISPR/Cas9 constructs and further mating assays. The vegetative mycelia of L. edodes were cultured on potato dextrose agar (PDA) and grown at 25 °C in the absence of light.

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| gRNA name | Forward sequence (5′ to 3′) | Reverse sequence (5′ to 3′) |
|----------|---------------------------|---------------------------|
| gRNA-1   | GATTGCTCGTACTGATCCGTC    | AAACGTACCGATGAGTCGCGAGC  |
| gRNA-2   | GATTGACGCTTCGCTGACTTCTCTTCT | AAACAGAAGGCTACAGAAGAATGC |
| gRNA-3   | GATTCTGGAGATTGAAAATCCCAAGA | AAACCTTGATTTGAAAACCTACAC |
| gRNA-4   | GATTCTCGTCTCCCAACTCCCAAG  | AAACCTTGATTTGAAAACCTACAC |
| gRNA-5   | GATTGGAGATGGAGGATTTGACCTT | AAACAACTGGCAACTCCCTACCTAC |
| gRNA-6   | GATTGAGATGGAGGATTTGACCTT | AAACAACTGGCAACTCCCTACCTAC |
| gRNA-7   | GATTGCATCGCCTGCCCTCTTCTTAC | AAACACCTGGTCCCTCCCTACAC |
| gRNA-8   | GATTGGTGGAGGAGCAGGGGTGTT  | AAACACCTGGTCCCTCCCTACAC |

Additional sequences for ligation with linearized vector by AarI are highlighted in bold.

Table 1. DNA sequences of gRNA spacer used in this study.
mycelia were examined under a Nikon Eclipse Ti-U (or Photofluor LM-75 for fluorescence) inverted microscope. Images were captured using a Nikon DS-Ri2 camera with NIS Elements software.

*L. edodes* was grown in 50 mL of PDB for 10 d at 25°C. The mycelia were collected by centrifugation for 10 min and were ground in liquid nitrogen to obtain a mycelial powder. Total RNA was extracted from the ground mycelia using the TaKaRa MiniBEST Universal RNA Extraction Kit (Takara Bio, Shiga, Japan). The obtained RNA was used for cDNA synthesis using the TOPscript cDNA Synthesis Kit (Enzynomics, Daejeon, Korea). Real-time PCR (qPCR) analyses of the target gene were performed using FastStart Essential DNA Green Master (Roche, Basel, Switzerland) and specific primer sets [15]. The qPCR conditions were as follows: 5 min at 95°C for initial denaturation followed by 40 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C. Relative expression was calculated based on the difference in Cq value (2^(-ΔΔCq)) between a target gene and a reference gene (*β*-tubulin).

To characterize the *LeU6* promoter, a region conserved between the human and yeast *RNU6* gene was examined in the B17 monokaryon genome [16]. Five putative *RNU6* sequences (*LeU6*-2, -3, -5, -8, and -9) were identified, and the sequences are highly conserved (Figure 1(A)). To identify an effective *U6* promoter, the region 500 bp upstream of the *LeU6* transcription start site (TSS) was examined for elements such as a TATA-box. Two out of five potential promoters (*pleU6*-8 and *pleU6*-9) contain TATA-box-like consensus sequences (5’-TATA(A/T)A(A/T)-3’) around the −50 bp range (Figure 1(B)). Therefore, *pleU6*-9 was chosen as the promoter for sgRNA expression.

Using the identified *LeU6* promoter, we constructed a simple CRISPR/Cas9 genome editing plasmid vector system (Figure 2(A)). To properly express the Cas9 protein, we used a constitutively active *Legpd* promoter [14]. The mating type genes in *L. edodes*, a heterothallic basidiomycete, consist of two unlinked loci, identified as *LeAs* (HD1 and HD2) and *LeBs* [17,18]. We targeted the gene *LeHD1* for genome editing, designed eight gRNAs, and constructed sgRNA expression vectors (Figure 2(B)). We then pooled the resultant eight vectors into two groups and transformed them into the protoplasts of monokaryotic *L. edodes* (SJ 705). To select for transformed protoplasts, we grew the regenerated transformants on hygromycin-containing PDA plates. The genomes of the selected transformants were further confirmed after PCR amplification of the *HPT* gene, and Sanger sequencing analysis of the sgRNA target regions was performed (Figure 2(C)). Over 100 transformants were selected and initially screened using the PCR product size of the *HD1* gene (Figure 3(A)). Among the tested transformants, we selected two *CR-Lehd1* mutants with a slightly smaller PCR product size (*hd1*-1 and *hd1*-2) (Figure 3(A)). Sanger sequencing identified a 2-bp (*hd1*-1) and 33-bp (*hd1*-2) deletion mutation for the two *hd1* alleles (Figure 3(B)), which indicates that successful genome editing by the CRSPR/Cas9 system with three active gRNAs (gRNA1, 2 and 7) occurred in the *L. edodes* genome.

For proper mating of two comparative monokaryotic strains of *L. edodes*, the heterodimeric HD1/2 complex controls downstream events including nuclear pairing, clamp connection, and coordination of nuclear division [19]. Because we successfully obtained two putative loss-of-function alleles of *HD1*, we next tested the mating processes of the *hd1* mutants with a comparative monokaryotic strain (SJ705-3). Surprisingly, all of the loss-of-function *CR-hd1* mutants exhibited a normal mating phenotype with well-established clamp connections.
However, the downstream target genes of the HD1/2 complex in the mating processes, including CLP1 and priA, were significantly downregulated in the hd1-1 mutant mycelial tissue (Figure 3(D)). The 2-bp deletion in the hd1-1 mutant also resulted in the suppression of HD1...
transcription, but not that of HD2 or ZNF2 (Figure 3(D)). These results suggest that the transcription of critical mating-related genes was impaired by the CRISPR/Cas9-mediated deletion mutation in the HD1 gene. In addition, other mating-related systems are likely integrated into the canonical HD1/2-mediated transcriptional network to ensure proper mating occurs in L. edodes.

In this study, we present the first development of an efficient CRISPR/Cas9-based genome editing tool kit for the important edible mushroom, L. edodes. The identified endogenous LeU6 promoter drives the expression of sgRNAs in L. edodes cells, and the Cas9 proteins are targeted to the sgRNA genome binding sites to produce double-strand breaks (DSBs). In addition, our PEG-mediated protoplast transformation system can be efficiently used for the high throughput screening of active sgRNAs in this mushroom. Based on the system reported in this study, future studies can focus on developing marker-free gene mutagenesis using a Cas9/sgRNA ribonucleoprotein (RNP) complex. Furthermore, CRISPR/Cas9-assisted gene replacement via homology-directed repairs could be used for proper gene insertion into the genome. These biotechnological approaches will be necessary for the future development of mushroom breeding technologies.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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