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RESEARCH NOTE

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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 (shiitake) 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*.

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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

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Table 1. DNA sequences of gRNA spacer used in this study.

gRNA name	Forward sequence (5′ to 3′)	Reverse sequence (5′ to 3′)
gRNA-1	**GATT**GCTCGCTAGCTATCGGTGTCA	**AAAC**TGACACCGATAGCTAGCGAGC
gRNA-2	**GATT**GACTTCTCTGTGATGCCTTCT	**AAAC**AGAAGGCATCACAGAGAAGTC
gRNA-3	**GATT**GTTGTAGTTGTAAATCCAAGA	**AAAC**TCTTGGATTTACAACTACAAC
gRNA-4	**GATT**GTCCTCCTCCCAACTCCCAAG	**AAAC**CTTGGGAGTTGGGAGGAGGAC
gRNA-5	**GATT**GAGGATGGGAGGTTGCCACTT	**AAAC**AAGTGGCAACCTCCCATCCTC
gRNA-6	**GATT**GTAGGATGGGAGGTTGCCACT	**AAAC**AGTGGCAACCTCCCATCCTAC
gRNA-7	**GATT**GACATCCCGCCCTCCTACACC	**AAAC**GGTGTAGGAGGGCGGGATGTC
gRNA-8	**GATT**GTTGGAGAGGAGCCAGGTGT	**AAAC**ACACCTGGCTCCTCTCCAAC

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

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 RNU6-1* (GenBank X07425.1) was used as the query to search for homologs in the *L. edodes* genomic 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 RNU6-1* (*hRNU6-1*). Sequence comparison between *hRNU6-1* and *LeU6*s 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 (Enzynomics, Daejeon, Korea). The backbone plasmid, pHAtC, was acquired from Addgene (plasmid # 78098). The promoter sequences for *L. edodes glyceraldehyde-3-phosphate dehydrogenase* (*gpd*) and *U6* were amplified using the primer pairs *pLegpd*-F (5′-AAGGCCTTCGATATCAGTCAGA TTGTCA-3′) with *pLegpd*-R (5′-GACTAGTGGCCTGAATAGACATGGA AT-3′) and *pLeU6*-F (5′-GGAATTCCACTTCGCCG ATGCGAAATA-3′) with *pLeU6*-R (5′-CCGCTC GAGACACCTGCCTCCAATCATTGATGAACTACT-TAACAATCAG-3′), respectively. After the *U6* promoter was cloned into the *Xho*I–*Eco*RI site of pHAtC, the plasmid was linearized using *Stu*I and *Spe*I, and the *gpd* promoter sequence was ligated between these restriction sites. The annealed oligo DNA fragments corresponding to the *HD1* gRNA target were inserted into the *Aar*I site between *pLeU6* and the gRNA scaffold sequence to yield a sgRNA/Cas9 binary vector for use in *L. edodes*.

Isolation and transformation of *L. edodes* protoplasts were performed as described previously [14]. Briefly, the mycelia cultured in potato dextrose broth (PDB) for 10 days were digested in a 2.5% Lysing Enzyme (Sigma-Aldrich, St. Louis, MO, USA) solution to isolate protoplasts. The protoplasts were suspended in 0.6 M sucrose and adjusted to a final concentration of approximately 10^7 cells/mL. For fungal transformation, 10^6 protoplast cells were incubated with 20 µg plasmid DNA on ice for 5 min. Next, 100 µL of PTC solution (40% polyethylene glycol 4000, 10 mM Tris-HCl, pH 7.5, and 50 mM $CaCl_2$) was added. After incubation at room temperature for 5 min, 600 µL of 0.6 M sucrose was added and mixed by inverting. Plasmids containing eight different guide DNA sequences were pooled into two groups (gRNA-1, gRNA-2, and gRNA-3 or gRNA-4, gRNA-5, gRNA-6, gRNA-7, and gRNA-8) and used for transformation. After a month of incubation on regeneration plates, each transformed mycelium was isolated and cultured on PDA for 10 d. Transformed protoplasts were spread on regeneration plates (PDA containing 0.6 M sucrose and 30 µg/mL hygromycin B). Genomic DNA extracted from each transformant was used as a template for amplification of the *hygromycin phosphotransferase* (*hpt*) and *HD1* genes. The primer pairs *HPT*-F (5′-GATGCCTCCGCTCGAAGTAGCG-3′) with *HPT*-R (5′-GCATCTCCCGCCGTGCAC-3′) and *HD1*-F (5′-TCGTCTACTCACCTCCCTCG-3′) with *HD1*-R (5′-TCCGCGTCTTATACTTCGGC-3′) were used for *HPT* or *HD1* amplification, respectively. The PCR products for *HD1* from each transformant were analyzed using Sanger sequencing.

To confirm the mating phenotype of *hd1*-Cas9 strains, PDA was used for cocultivation with a paired mating strain (SJ705-3). Sections of agar containing hyphae were placed close to each other on PDA plates and incubated at 25 °C for 2–3 weeks.

For microscopic analysis, the dikaryotic mycelia produced were scraped into 100 µL of water and were disrupted by vortexing. The mycelia were stained with an equivalent mixture of 10% potassium hydroxide and calcofluor white (Sigma-Aldrich). The stained

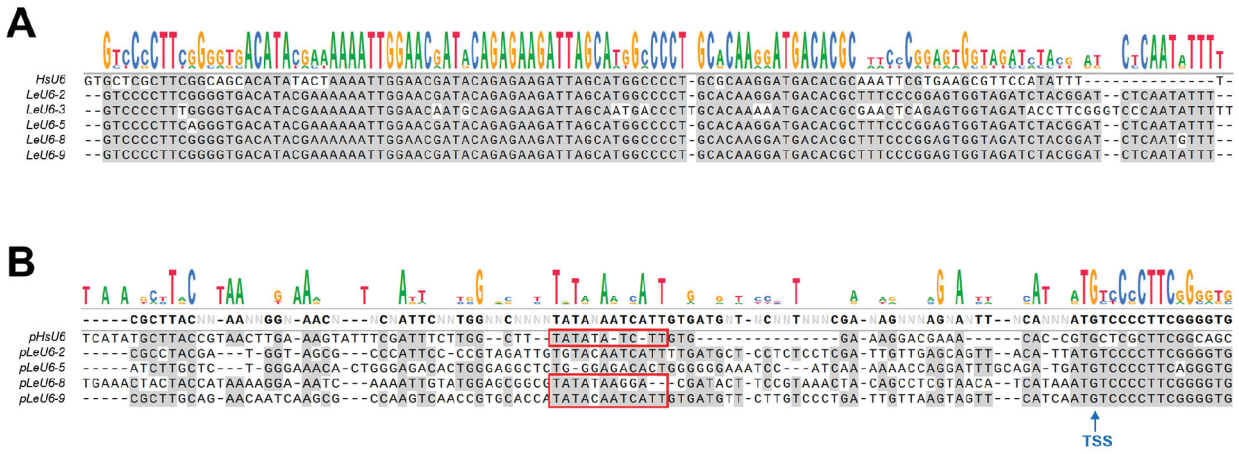

Figure 1. Comparison between human *U6* and *Lentinula edodes U6* small nuclear RNAs. (A) Multiple sequence alignment between human and *L. edodes U6* gene. *HsU6*, human *U6*. 5 highly-conserved sequences were identified in scaffold 2, 3, 5, 8 and 9 in B17 genome [16]. (B) Putative TATA box in p*LeU-8* and *pLeU-9* promoter region (red box). TSS, Transcriptional Start Site (blue arrow).

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 ($2^{-\Delta\Delta Cq}$) 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 sgRNAs (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

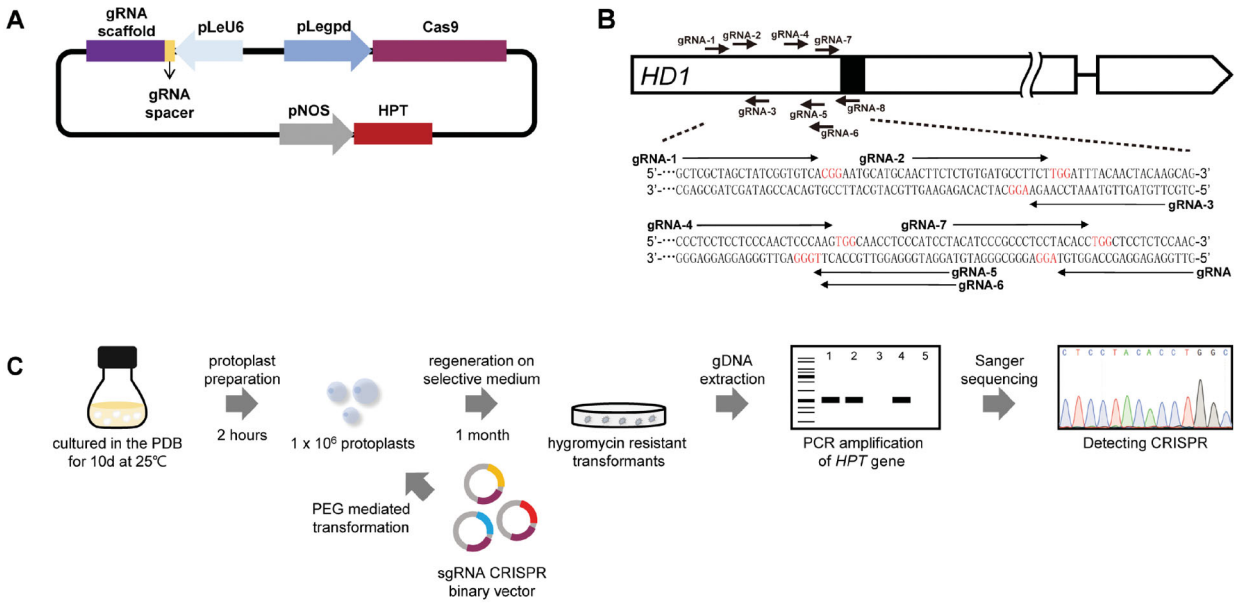

Figure 2. Establishment of CRISPR/Cas9 gene editing system through plasmid DNA transformation in *Lentinula edodes* protoplasts. (A) Schematic diagram of the sgRNA/Cas9 binary vector. *pLegpd*, promoter of a *glycerol-3-phosphate dehydrogenase* gene from *L. edodes*. *pNOS*, promoter of a *nospaline synthase* gene. HPT, the *hygromycin phosphotransferase* gene. (B) Target sites for eight sgRNAs targeting *HD1*. The gene structure (upper) and partial abbreviate sequence (lower) of *HD1* were shown. The homeodomain and sgRNAs were represented as a black box and arrows, respectively. PAM sites (5′-NGG-3′) were indicated red at the end of each spacer sequence. (C) Experimental design and workflow of the CRISPR/Cas9 gene-editing *via* PEG mediated DNA transformation.

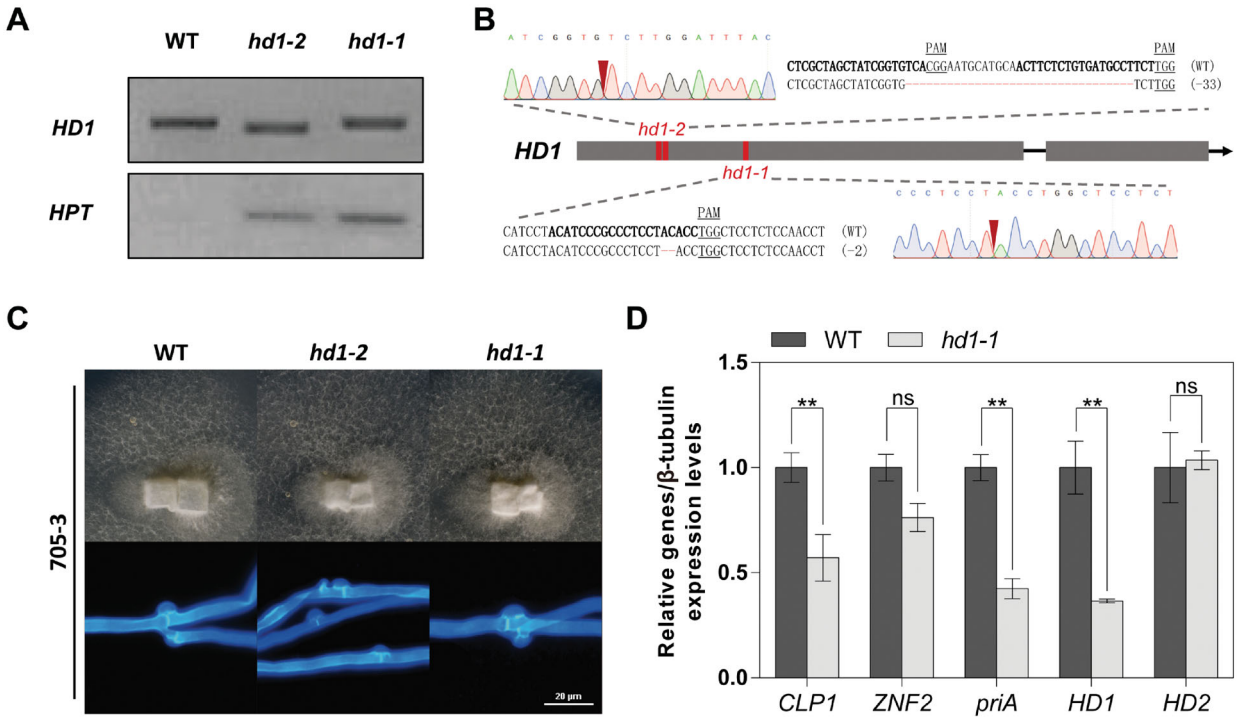

Figure 3. Targeted mutagenesis of *HD1* gene using CRISPR/Cas9 system. (A) PCR amplification of *HD1* and *HPT* gene in genomic DNA of wild type and transformants, *hd1-2* and *hd1-1*. (B) Sanger sequencing of the wild type and *HD1* transformants. Locations that cleaved by CAS9 endonuclease and mutated were shown in red boxes and arrowheads. Dashes indicate deleted nucleotides, bolded and underlined sequences represent spacer sequence and PAM motif of sgRNA, respectively. (C) Mating assay and dikaryotic clamp cell connection of *hd1* mutants. Dikaryotic hyphae were stained with a calcofluor white. Scale bar = 20um. (D) Real-time qRT-PCR analysis of *CLP1*, *ZNF2*, *priA*, *HD1* and *HD2* transcript levels in the *hd1-1* mutant. Error bars indicate SE of the mean ($n = 3$; $^{**}p < 0.01$; Student's *t*-test).

(Figure 3(C)). 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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