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CRISPR-Cas9 assisted functional gene editing in the mushroom *Ganoderma lucidum*

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Abstract

The genetic manipulation of basidiomycete mushrooms is notoriously difficult and immature, and there is a lack of research reports on clustered regularly interspaced short palindromic repeat (CRISPR) based gene editing of functional genes in mushrooms. In this work, *Ganoderma lucidum*, a famous traditional medicinal basidiomycete mushroom, which produces a type of unique triterpenoid-anti-tumor ganoderic acids (GAs), was used, and a CRISPR/CRISPR-associated protein-9 nuclease (Cas9) editing system for functional genes of GA biosynthesis was constructed in the mushroom. As proof of concept, the effect of different gRNA constructs with endogenous *u6* promoter and self-cleaving ribozyme HDV on *ura3* disruption efficiency was investigated at first. The established system was applied to edit a cytochrome P450 monooxygenase (CYP450) gene *cyp5150l8*, which is responsible for a three-step biotransformation of lanosterol at C-26 to ganoderic acid 3-hydroxy-lanosta-8, 24-dien-26 oic acid. As a result, precisely edited *cyp5150l8* disruptants were obtained after sequencing confirmation. The fermentation products of the wild type (WT) and *cyp5150l8* disruptant were analyzed, and a significant decrease in the titer of four identified GAs was found in the mutant compared to WT. Another CYP gene involved in the biosynthesis of squalene-type triterpenoid 2, 3; 22, 23-squalene dioxide, *cyp505d13*, was also disrupted using the established CRISPR-Cas9 based gene editing platform of *G. lucidum*. The work will be helpful to strain molecular breeding and biotechnological applications of *G. lucidum* and other basidiomycete mushrooms.

Keywords *Ganoderma lucidum* · CRISPR-Cas9 · Genome editing · CYP450 · Triterpenoid biosynthesis · Medicinal mushroom

Introduction

Mushrooms have broad applications in functional food, pharmaceutical, and healthcare industries due to their rich valuable secondary metabolites (Zhong and Tang 2004). *Ganoderma lucidum* (Lingzhi in Chinese, Reishi in Japanese), a kind of famous traditional medicinal mushroom in East Asia, occupies a large market and produces a group of highly oxygenated lanostane-type triterpenoids-ganoderic acids (GAs). GAs have been proved to possess important biological and pharmaceutical activities, including anti-tumor and anti-metastasis activities (Gill et al. 2016; Sato et al. 2009; Xu et al. 2010a, b, c), and their efficient bioproduction has received great interest (Xu et al. 2013). In our recent work, heterologous expression of *cyp5150l8* from *G. lucidum* in a brewing yeast led to a three-step oxidation of lanosterol at C-26 to produce an anti-tumor GA, 3-hydroxy-lanosta-8, 24-dien-26 oic acid (HLDOA) (Wang et al. 2018). However, the original biosynthetic pathway from lanosterol to GAs is yet unclear, in which the involved cytochrome P450s (CYP450s) for the GA biosynthesis post-modification have not been characterized in the mushroom. The problems of unclear biosynthetic pathway and unknown biosynthesis genes severely hinder the cell factory engineering of *Ganoderma* to further improve the GA production (Xiao and Zhong 2016).

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In a few basidiomycete mushrooms such as *Schizophyllum commune* (Ohm et al. 2010) and *Coprinopsis cinerea* (Nakazawa et al. 2011), the traditional homologous recombination based gene disruption technology was applied to functional gene analysis, but this genetic tool is still not easy for applying to other basidiomycete mushrooms. Recently, CRISPR-Cas9 based gene editing, as the new third generation of genome editing technology, has been well applied to various species, including bacteria, animal and plant cells. Unfortunately, until now, there have been only several pieces of news on CRISPR-Cas9 based gene editing of a functional gene in a mushroom, i.e., *ppo* gene encoding polyphenol oxidase in *Agaricus bisporus* (Hall 2016; Schuster and Kahmann 2019; Waltz 2016a, 2016b), but no research papers or patents related to the news report is available. Recently, in *S. commune*, a homeodomain transcription factor gene *hom2* was disrupted by preassembled Cas9 ribonucleoproteins (RNPs) (Vonk et al. 2019), but its editing efficiency was very low (0.15–1.8 mutants obtained from 10^7 protoplasts) and RNPs preparation was a time-consuming and costly process. The above facts reflect the difficulty of functional gene studies in basidiomycete mushrooms, not to mention GAs of *G. lucidum*. Therefore, establishment of a functional gene editing technology is urgently required for basidiomycete mushrooms to promote their biology research and biotechnology development.

Recently, a CRISPR-Cas9 based gene disruption of a marker gene *ura3* in *G. lucidum* was established, with an efficiency of 0.2–1.78 mutants per 10^7 protoplasts (Qin et al. 2017). However, this technology has several limitations: (i) the high cost and tedious process for preparation of in vitro expressed guide RNA (gRNA), and (ii) gene disruption by sequenced delivery of Cas9 and in vitro transcribed gRNA, making the second round selection of gRNA containing strains unavailable. As a result, it is unfeasible to adopt this method to obtain a functional gene disruption mutant of *G. lucidum*. To overcome this problem, development of a CRISPR-Cas9 system enabling in vivo expression of gRNA is a promising alternative, in which a promoter capable of driving gRNA expression in *G. lucidum* should be identified at first.

Until now, two types of promoters have been reported for in vivo expression of gRNA in eukaryotes. One is transcribed by RNA polymerase II (pol II), which needs an additional ribozyme, e.g., of hammerhead (HH) type and hepatitis delta virus (HDV), to mediate gRNA cleavage to precisely release gRNA (Gao and Zhao 2014). Another is transcribed by RNA polymerase III (pol III), such as *u3* and *u6* promoter, which is used in mammalian cells (Cong et al. 2013), crop plants (Shan et al. 2013), a rice blast fungus *Pyricularia oryzae* (Arazoe et al. 2015), a fungal maize pathogen *Ustilago maydis* (Schuster et al. 2016), and a mushroom *Coprinopsis cinerea* (Sugano et al. 2017). Since these gene sequences are conserved in eukaryotes (Brow and Guthrie 1988; Sun et al. 2015) (Supplemental Fig. S1A), their promoters may be taken as candidates for expression of gRNA in *G. lucidum*. In addition, it is noted that the pol III promoters including *u6* promoter adopt a T-stretch as termination signal, yielding small RNA transcripts with different lengths of U-tails (Gao et al. 2018a), and those U-tails may have a negative effect on functionality of CRISPR system (Gao et al. 2018a). It is our hypothesis that use of cleaving ribozyme HDV to keep a correct transcript size by pol III promoters may enhance the gene editing efficiency of CRIPSR-Cas.

In this work, by identifying a suitable *u6* promoter for in vivo expression of gRNA, a CRISPR-Cas9 system was developed to disrupt a marker gene (*ura3*) in *G. lucidum*, and incorporation of an HDV ribozyme at the 3′ end of gRNA was also adopted to improve the gene editing. This established technology was then applied to precisely edit a functional gene *cyp5150l8* via CRISPR-Cas9 assisted homologous recombination (HR). The study is considered valuable to further gene editing and metabolic engineering of *G. lucidum* and other basidiomycete mushrooms.

Materials and methods

Strains and culture conditions

Escherichia coli strain DH5a (Tsingke, Beijing, China) was used as a cloning host. *G. lucidum* strain CGMCC 5.616 from China General Microbiological Fermentation Center was maintained on potato dextrose agar and used as the recipient strain for transformation. Cultivation of *G. lucidum* was performed as described previously (Xu et al. 2012). Briefly, for the initial preculture, mycelia from a slant test tube were inoculated into a shake flask and incubated for 5 days, then 5 ml of the precultured broth was transferred into next shake flasks, which were incubated for 3 days before being used as seed culture for static liquid culture. Five milliliters of the abovementioned seed culture was inoculated into a flat bottle containing 120 mL medium. The flat bottle was hand-shaken every 12 h to scatter the mycelia, and the cells were collected for preparation of protoplasts after 36 h. The complete yeast extract (CYM) medium (1% maltose, 2% glucose, 0.2% yeast extract, 0.2% tryptone, 0.05% MgSO4, 0.46% KH_2PO4, 0.6 M mannitol, and 1% agar) was used for protoplast cultivation as reported (Qin et al. 2017).

Sequencing of *ura3* and *cyp5150l8* from *G. lucidum* CGMCC 5.616

Using the genomic DNA of *G. lucidum* CGMCC 5.616 as template, *ura3* and *cyp5150l8* were amplified by primer pairs *URA3* F / *URA3* R (Supplemental Table S1) and *CYP5150L8* F/*CYP5150L8* R (Supplemental Table S1), cloned into the

pClone007 vector by using a pClone007 blunt simple vector kit (Tsingke, Beijing, China), transformed into *E. coli* DH5a (Tsingke, Beijing, China), and cultured on Luria-Bertani (LB) agar plates containing 100 μg/mL ampicillin, respectively. Then, 10 *E. coli* clones were randomly picked for sequencing by using primer M13F (Supplemental Table S1) to confirm whether single nucleotide polymorphisms (SNPs) were contained or not.

For the extraction of genomic DNA, 20 mg hypha were collected into a tube containing 10 small magnetic beads (Jingxin, Shanghai, China), 300 μL DES buffer (1% SDS, 2% Triton-100, 0.372% EDTA-2Na, 0.585% NaCl, 10 mM Tris–HCL at pH 8.0), and 300 μL hydroxybenzene-chloroform-isoamyl alcohol (24:25:1, pH 8.0, Dingguo, Beijing, China). The hypha were grinded for 5 min at 60 Hz by a tissue grinder (Jingxin, Shanghai, China) and subjected to centrifugation for 10 min at 12,000×*g* at 4 °C. The supernatant was transferred to a new tube containing 300 μL isopropanol and kept at − 20 °C for 30 min. Then, it was centrifuged for another 10 min at 12,000×*g* at 4 °C, then the supernatant was discarded. The pellets were resuspended in 300 μL precooled 75% ethanol and subjected to centrifugation for 10 min at 12,000×*g* at 4 °C, and the supernatant was discarded. The DNA pellets were dried for 10 min at 50 °C and resuspended in 200 μL distilled water. The DNA concentration was determined by the NanoDrop™ 2000 spectrophotometer (Thermo Scientific, Massachusetts, USA).

Construction of plasmids

For the construction of *ura3* disruption plasmids, *ura3.b* (Qin et al. 2017) was chosen as the targeting site. The sequences of p*U6-3-ura3.b*-gRNA-HDV and p*U6-4-ura3.b*-gRNA-HDV were synthesized and linked into the pUC57 vector (http://www.addgene.org/vector-database/4509/) by Ruimian biotech company (Shanghai, China) to yield plasmids pUC57-p*U6-3-ura3.b*-gRNA-HDV and pUC57-p*U6-4-ura3.b*-gRNA-HDV, respectively. The p*U6-3-ura3.b*-gRNA and p*U6-4-ura3.b*-gRNA were cloned by primer pairs pU6-3 F/gRNA-R (Supplemental Table S1) and pU6-4 F / gRNA-R (Supplemental Table S1) using pUC57-p*U6-3-ura3.b*-gRNA-HDV and pUC57-p*U6-4-ura3.b*-gRNA-HDV as template, respectively. The p*U6-3-ura3.b*-gRNA-HDV was amplified by primer pairs pU6-3 F /HDV R (Supplemental Table S1) using pUC57-p*U6-3-ura3.b*-gRNA-HDV as template. Plasmid pMD-Glcas9 (Qin et al. 2017), which contains a mutated *sdhB* gene (encoding the iron-sulfur protein subunit of succinate dehydrogenase) conferring carboxin resistance (Xu et al. 2012) was linearized by *Not*I (NEB, Beijing, China). The sequence of p*U6-3-ura3.b*-gRNA and linearized pMD-Glcas9 (Qin et al. 2017) was ligated to produce plasmid pMD-p*U6-3-ura3.b*-gRNA-Glcas9 (pU6-3-*ura3.b*-gRNA; Fig. 1a) according to the procedure described in the SoSoo cloning kit (Tsingke, Beijing, China). Similarly, the sequence of p*U6-4-ura3.b*-gRNA and p*U6-3-ura3.b*-gRNA-HDV was respectively ligated with linearized pMD-Glcas9 (Qin et al. 2017) to yield plasmid pMD-p*U6-4-ura3.b*-gRNA-Glcas9 (p*U6-4-ura3.b*-gRNA; Fig. 1c) and pMD-p*U6-3-ura3.b*-gRNA-HDV-Glcas9 (p*U6-3-ura3.b*-gRNA-HDV; Fig. 2a), respectively.

For the construction of *cyp5150l8* disruption plasmid pMD-p*U6-3-tl8*-gRNA-HDV-Glcas9 (Fig. 3a), and *cyp505d13* disruption plasmid pMD-p*U6-3-td13*-gRNA-HDV-Glcas9 (Fig. 5a), the sequences *tl8*-gRNA-HDV-Glcas9 and *td13*-gRNA-HDV-Glcas9 were firstly cloned by primer pair *CYP5150L8s*F/mGlCas9R (Supplemental Table S1) and *CYP505D13s*F/ mGlCas9R (Supplemental Table S1) using the plasmid p*U6-3-ura3.b*-gRNA-HDV as template. Then, the Glcas9-T*pdc-sdhB*-p*U6-3* sequence was cloned by primer pair mGlcas9 R F/PU6-3R using p*U6-3-ura3.b*-gRNA-HDV as template. The sequences of *tl8*-gRNA-HDV-Glcas9 and *td13*-gRNA-HDV-Glcas9 were ligated with Glcas9-T*pdc-sdhB*-p*U6-3* to yield plasmids pMD-p*U6-3-tl8*-gRNA-HDV-Glcas9 and pMD-p*U6-3-td13*-gRNA-HDV-Glcas9, respectively, according to the procedure described in the SoSoo cloning kit (Tsingke, Beijing, China).

To construct the donor plasmid for *cyp5150l8* disruption (Fig. 3b), the left and right homologous recombination arms were cloned by primer pairs L8D F1/L8D R1 (Supplemental Table S1) and L8DF2/L8DR2 (Supplemental Table S1) using genomic DNA of *G. lucidum* 5.616 as template, respectively. These two PCR products were ligated into a linearized pMD-18T (Takara, Dalian, China) to yield plasmid pMD-L8D (Fig. 3b) according to the procedure described in SoSoo cloning kit (Tsingke, Beijing, China). The PCR donor (L8D) (Fig. 3b) for *cyp5150l8* disruption was amplified by primer pair DL8F/DL8R (Supplemental Table S1) using pMD-L8D as template.

PEG-mediated transformation of protoplasts

The protoplast preparation and transformation were performed as reported (Qin et al. 2017). Briefly, the mycelia were collected and washed by 0.6 M mannitol and digested with 2% (w/v) lywallzyme for 2.5 h. Then, the obtained protoplasts were used for transformation as follows: about 10^7 protoplasts in 160 μL MTC buffer (0.6 M mannitol, 100 mM $CaCl_2$ and 100 mM Tris–HCl at pH 7.5) were transformed with 20 μg Cas9 plasmid, 3 μg donor (when needed), 10 μL 20 mM aurintricarboxylic acid (ATA), 5 μL 50 mM spermidine, 2 μL 50 mg/mL heparin, and 60 μL PTC buffer (40% polyethylene glycol (PEG)3350, 100 mM $CaCl_2$, 10 mM Tris-HCl at pH 7.5), then incubated on ice for 30 min. One milliliter of PTC buffer was added and incubated for 30 min at 28 °C. Later, the protoplasts were centrifuged for 5 min at 1575×*g* and resuspended in 1 mL CYM medium.

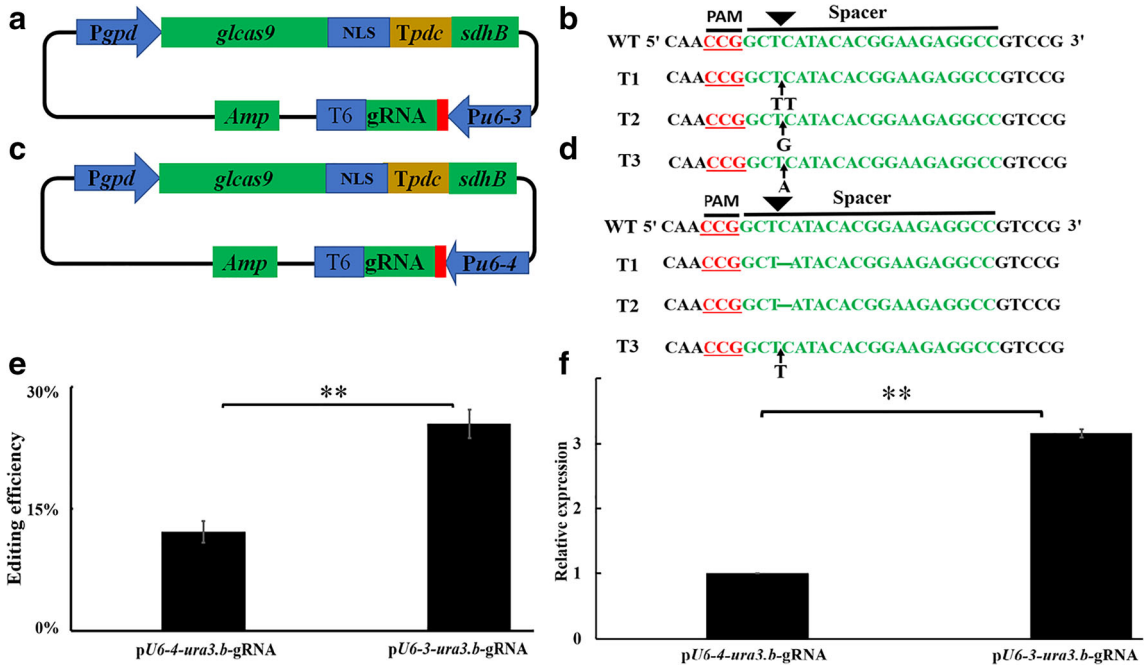

Fig. 1 Effect of *u6* promoters on *ura3* disruption efficiency. **a** p*U6-3-ura3.b*-gRNA. **b** Sequence alignment of three random mutants edited by p*U6-3-ura3.b*-gRNA. **c** p*U6-4-ura3.b*-gRNA. **d** Sequence alignment of three random mutants edited by p*U6-4-ura3.b*-gRNA. **e** The editing results of *ura3*. Editing efficiency was the ratio of *ura3* disruptants to carboxin resistant transformants. **f** Relative expression of gRNA in *G. lucidum* protoplasts transformed with p*U6-3-ura3.b*-gRNA as compared to those with p*U6-4-ura3.b*-gRNA. **a**, **c** Red box represents *ura3* spacer-*ura3.b*: GGCCTCTTCCGTGTATGAGC (5′-3′), PAM was "CGG (5′-3′)". **b**, **d** PAM is shown in red, black triangle represents cutting site of Cas9, spacer is shown in green, black arrows denote an insertion, short green lines denote different deletions. $^{**}P < 0.01$

RNA extraction and qPCR

Protoplasts transformed with various disruption plasmids were firstly cultured in 1 mL CYM medium for 6 h. Next, carboxin was added into the medium at a final concentration of 4 mg/L. After another 42 h cultivation, total RNAs were extracted from about 1.5×10^7 protoplasts using the TRIzol reagent (Sangon, Shanghai, China) according to the user's instruction. For qPCR, the DNase I treated total RNA was reverse-transcribed to produce cDNAs using primer oligo

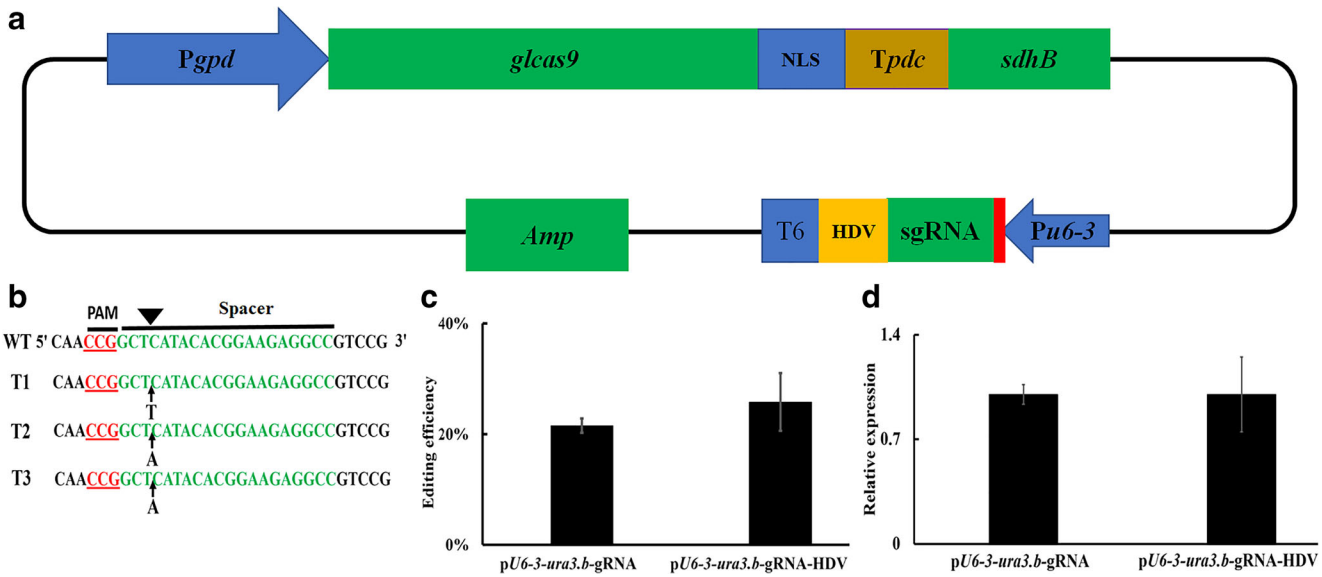

Fig. 2 Effect of HDV on *ura3* disruption efficiency. **a** p*U6-3-ura3.b*-gRNA-HDV; red box represents *ura3.b*: GGCCTCTTCCGTGTATGAGC (5′-3′). **b** Sequence alignment of three random mutants edited by p*U6-3-ura3.b*-gRNA-HDV; PAM is shown in red, black triangle represents cutting site of Cas9, spacer is shown in green, black arrows denote insertions. **c** The editing results of *ura3*. **d** Relative expression of gRNA in *G. lucidum* protoplasts transformed with p*U6-3-ura3.b*-gRNA-HDV as compared to those with p*U6-3-ura3.b*-gRNA

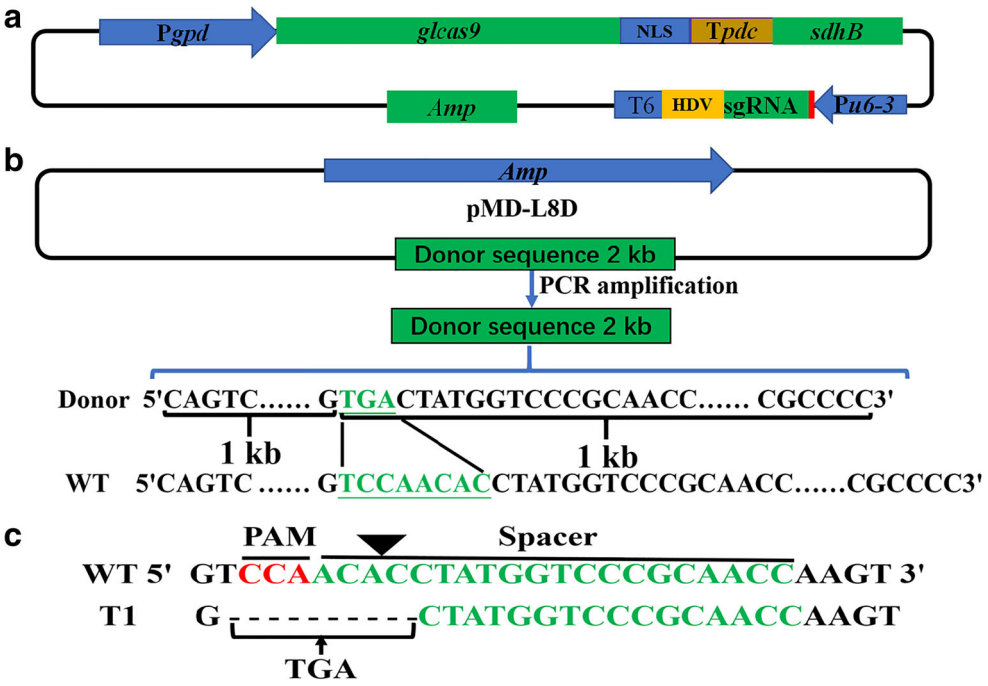

Fig. 3 Editing of *cyp5150l8* in *G. lucidum*. **a** A *cyp5150l8* editing plasmid, p*U6-3-tl8*-gRNA-HDV. Red box represents *cyp5150l8* spacer (*tl8*): GGTTGCGGGACCATA GGTGT (5′-3′), PAM was "TGG (5′-3′)". **b** Donors for repair of *cyp5150l8*. **c** Alignment of *cyp5150l8* homozygous mutant and WT. PAM is shown in red, black triangle represents cutting site of Cas9, target sequence (spacer) is shown in green, and black arrows and short black lines denote insertions and deletions, respectively

(dT) (Thermo Scientific, Massachusetts, USA) and specific primer gRNA-R (Supplemental Table S1) with Muumuu (Thermo Scientific, Massachusetts, USA) according to the manufacturer's instructions. The qPCR was performed with Luna qPCR mix (New England Bilabials, Beijing, China). The primer pair gRNA-F1/gRNA-R1 (Supplemental Table S1) was used for the amplification of gRNA of *ura3*. The philosophic 5 (*cyp5*) gene was used as the internal reference (Xu et al. 2015).

Selection of mutants

Fluoroscope acid (FOA)-resistant mutants were selected as follows: after transformation of *ura3* disruption plasmid and RNA extraction, the 10^7 protoplasts were poured onto solid CYM medium containing 4 mg/L carboxin for the first round of selection, and selected transformants were transferred to solid CYM medium containing 400 mg/L FOA for the second round of selection. Then, reselected transformants in FOA medium were confirmed by sequencing. After transformation of *cyp5150l8* disruption plasmid, protoplasts were cultured in 1 mL liquid CYM medium containing 20 μM 5,6-bis (benzylideneamino)-2-mercapto-pyrimidin-4-ol for 12 h, and then poured onto solid CYM medium containing 4 mg/L carboxin for verification.

Verification of transformants

To verify *ura3*, *cyp5150l8*, and *cyp505d13* disruptants, the target sequences were amplified by primer pairs *URA3* F/*URA3* R (Supplemental Table S1), *CYP5150L8* F/*CYP5150L8* R (Supplemental Table S1), and *CYP505D13* F/*CYP505D13* R (Supplemental Table S1) using genomic DNA of corresponding transformants as templates. Then, they were subjected to sequencing for confirmation.

As basidiomycete mushrooms including *G. lucidum* are dikaryotic, for the verification of the gene-edited transformants, it is necessary to select monokaryon *cyp5150l8* mutants. For this, protoplasts from the hyphae of dikaryotic mutants were prepared, and the hyphal cultivation and protoplast preparation were performed as reported (Qin et al. 2017). Protoplasts of 10^3 were cultured in 1 mL liquid CYM for 12 h and poured onto solid CYM medium containing 4 mg/L carboxin for selection. After 7 days, 20 clones were randomly picked out and transformed to a new solid CYM medium with 4 mg/L carboxin. The reselected transformants were verified as above mentioned. If the *cyp5150l8* editing region was unified and consistent with the PCR donor (L8D), the *cyp5150l8* sequence of corresponding mutant was cloned into pClone007 vector and sequencing confirmation was also done. If the sequencing results of all 10 clones were the same as L8D, the selected *cyp5150l8* mutant was also subjected for microscopic examination, as described below, to confirm whether it was a monokaryon.

The microscopic examination of mycelia

The mycelia were incubated in a shake flask at 28 °C for 48 h and subjected to Olympus CX43 microscope (Olympus, Tokyo, Japan) for detection of clam connection. For the observation of nuclei, about 10 mg mycelia were fixed with a 200 μL ethanol/acetic acid (3:1) solution for 30 min at 4 °C.

Then, the fixed hyphae were stained with 4,6-diamidino-2-phenylindole (DAPI) in 150 μL PBS buffer with 3.3 μg/mL final concentration for 10 min, washed twice with PBS buffer, and subjected to an Zeiss Axio Imager M2 fluorescence microscope (Zeiss, Jena, Germany) with a DAPI excitation apparatus.

Analyses of cell growth and GA

Fermentation of *G. lucidum* was performed as described earlier (Xu et al. 2013). The cells collected from mycelia mat and aerial mycelia was analyzed for their dry cell weight (DCW), and extraction of GA was performed as previously reported (Xu et al. 2013). The extracted GA samples were subjected to high-performance liquid chromatography (HPLC) (Agilent Technologies 1200 series, California, USA), equipped with a ZORBOX 300SB-C18 column (5 μm, 4.6 mm × 250 mm). The mobile phase A contained methanol/formic acid (100:0.1 v/v) and mobile phase B was 100% water. A linear gradient from 80% A to 100% A in 20 min at 1 mL/min was adopted.

Results

Disruption of *ura3* by identifying a suitable promoter for in vivo expression of gRNA in *G. lucidum*

To find a promoter capable of driving expression of gRNA in vivo, the *u6* gene transcribed by pol III promoter needed to be determined in *G. lucidum* at first. A highly conservative sequence was observed by sequence alignment with *u6* genes from *Schizosaccharomyces pombe* (GenBank No.X14196.1), *Trichophyton rubrum* (GenBank No.KC353131.1), *Aspergillus niger* (GenBank No.AY136823.1), *Arabidopsis thaliana* (Gen Bank No. X52527.1), and *Homo sapiens* (GenBank No.NR_004394.1) (Supplemental Fig. S1A). Five genes, named as *glu6a*, *glu6b*, *glu6c*, *glu6d*, and *glu6e* (Supplemental Fig. S1C), respectively, were predicted as *u6* genes (Supplemental Fig. S1B) by blasting this conservative sequence with the genomic sequence of *G. lucidum*, which was reported previously (Chen et al. 2012). The sequences starting after the stop codon of the last gene and ending before the predicted transcription initiation site of *u6* were accordingly selected as promoters and they were named as p*U6-0*, p*U6-1*, p*U6-2*, p*U6-3*, and p*U6-4*, respectively. Further, promoters p*U6-3* and p*U6-4* were randomly chosen to express gRNA in vivo (Fig. 1a, c). Since no SNPs was observed in the target region of *ura3* (Supplemental Fig. S2A), a codon-optimized Cas9 and a gRNA (site *ura3.b*) targeting 591 bp downstream of the start codon of *ura3* were adopted for testing *ura3* disruption efficiency here (Fig. 1a, c).

When transformed with plasmid p*U6-3-ura3.b*-gRNA, 6 FOA-resistant mutants were obtained from 28 carboxin-resistant transformants and the sequencing results from three randomly picked FOA-resistant mutants showed that small insertions were detected in the 3 bp upstream of protospacer-adjacent motif (PAM) (Fig. 1b), which was consistent with the cleavage site of Cas9 (Cong et al. 2013; Mali et al. 2013). For disruption of *ura3* using plasmid p*U6-4-ura3.b*-gRNA, three FOA-resistant mutants were obtained from 27 carboxin-resistant transformants and small insertions in the 3 bp upstream of PAM were also detected in three randomly picked FOA-resistant mutants (Fig. 1d). As a result, the *ura3* editing efficiency (ratio of *ura3* disruption mutants to carboxin-resistant transformants) using p*U6-3-ura3.b*-gRNA and p*U6-4-ura3.b*-gRNA was 21.5% and 11.4%, respectively (Fig. 1e), while a similar trend was observed from another set of experiments (Supplemental Table S2). Besides, the expression level of gRNA of protoplasts transformed with plasmid p*U6-3-ura3.b*-gRNA was 3.16-fold higher than that of protoplasts transformed with p*U6-4-ura3.b*-gRNA (Fig. 1f), which may be the reason for higher editing efficiency in the former case.

Effect of HDV on *ura3* disruption efficiency

As reported, a correct size of gRNA is critical for a high efficiency of CRISPR-Cas9 mediated gene editing (Gao and Zhao 2014; Zhang and Matlashewski 2015). To achieve precise transcriptional termination, here, the HDV ribozyme at the 3′ end of gRNA was incorporated, and the constructed p*U6-3-ura3.b*-gRNA-HDV disruption plasmid was used to test whether the *ura3* editing efficiency could be increased or not (Fig. 2a). As a result, a total of 8 FOA-resistant mutants were obtained from 31 carboxin-resistant transformants and small insertions were detected in 3 bp upstream of PAM in three randomly picked FOA-resistant mutants (Fig. 2b). The *ura3* editing efficiency with and without HDV were 25.8% and 21.5%, respectively (Fig. 2c). Meanwhile, the corresponding gRNA expression levels were almost the same (Fig. 2d). In another set of experiments, the same higher trend of *ura3* editing efficiency with p*U6-3-ura3.b*-gRNA-HDV against p*U6-3-ura3.b*-gRNA was obtained (32.0% v.s. 25.5%, $p < 0.05$, Supplemental Table S2).

Editing of two functional genes (*cyp5150l8* and *cyp505d13*) by applying the above-established CRISPR-Cas9

To construct a disruption plasmid for editing a functional gene *cyp5150l8*, the gRNA (site *tl8*) targeting 244 bp downstream of the start codon of *cyp5150l8* was chosen (Fig. 3a), where no SNPs were observed after sequencing confirmation (Supplemental Fig. S2B). Similar to the case of *ura3* editing,

addition of HDV also enhanced the *cyp5150l8* gene editing efficiency (data not shown). To achieve precise editing by HR rather than non-homologous end jointing (NHEJ), a 2 kb repair donor containing 1 kb upstream and downstream sequence close to CRISPR-Cas cleavage site was designed. Particularly, the 8 bp including PAM sequence "TCCAACAC" was replaced with "TGA" to introduce a stop codon for early translation termination of target protein (Fig. 3b). As a result, two *cyp5150l8* mutants were obtained from 68 transformants, but double peaks were detected around the CRISPR-Cas9 editing region according to the sequencing results (Supplemental Fig. S3), implying those mutants were heterozygous with only one copy nuclei edited. As *G. lucidum* is dikaryotic, to obtain a monokaryotic mutant, protoplasts of these two dikaryons were prepared and the regenerated strains were then obtained. As for a monokaryotic strain, only one nucleus existed between two adjacent septa (Herzog et al. 2016), and no clamp connection was observed (Yi et al. 2010). After sequencing confirmation (Fig. 4a) and microscopic examination (Figs. 4b–i), monokaryotic strains of both WT (WT-reg) and mutant (ML8-reg) were obtained.

The fermentation products of WT-reg and ML8-reg were analyzed by HPLC. A typical HPLC chromatogram and the mass spectrometry (MS) data of four individual GAs (GA-Mk, T, S, and Me) existing in both WT-reg and ML8-reg are shown in Supplemental Fig. S4. As shown in Fig. 5, there was not much difference in their cell growth (Fig. 5a), but the titer of four identified GAs (Fig. 5b), i.e., GA-Mk, T, S, and Me, was significantly decreased in the mutant ML8-reg compared to WT. For example, the titer of GA-Mk reduced to 0.01 mg/g DCW, much less than that of WT (1.33 mg/g DCW), and that of GA-T in WT was 5.56 mg/g DCW, but only 0.14 mg/g DCW in the mutant. Three direct catalytic products of CYP5150L8 from lanosterol (Wang et al. 2018), i.e., 3-hydroxy-lanosta-8, 24-dien-26-ol (HLDO), 3-hydroxy-lanosta-8, 24-dien-26-al (HLDA), and 3-hydroxy-lanosta-8, 24-dien-26 oic acid (HLDOA), were not identified in both WT and the mutant.

To test the editing efficiency of this system when targeting at other functional gene in *G. lucidum*, another CYP gene (*cyp505d13*) involved in the bioproduction of squalene-type triterpenoid 2,3; 22,23-squalene dioxide (Song et al. 2019) was chosen as the candidate. The gRNA (site *td13*) targeting

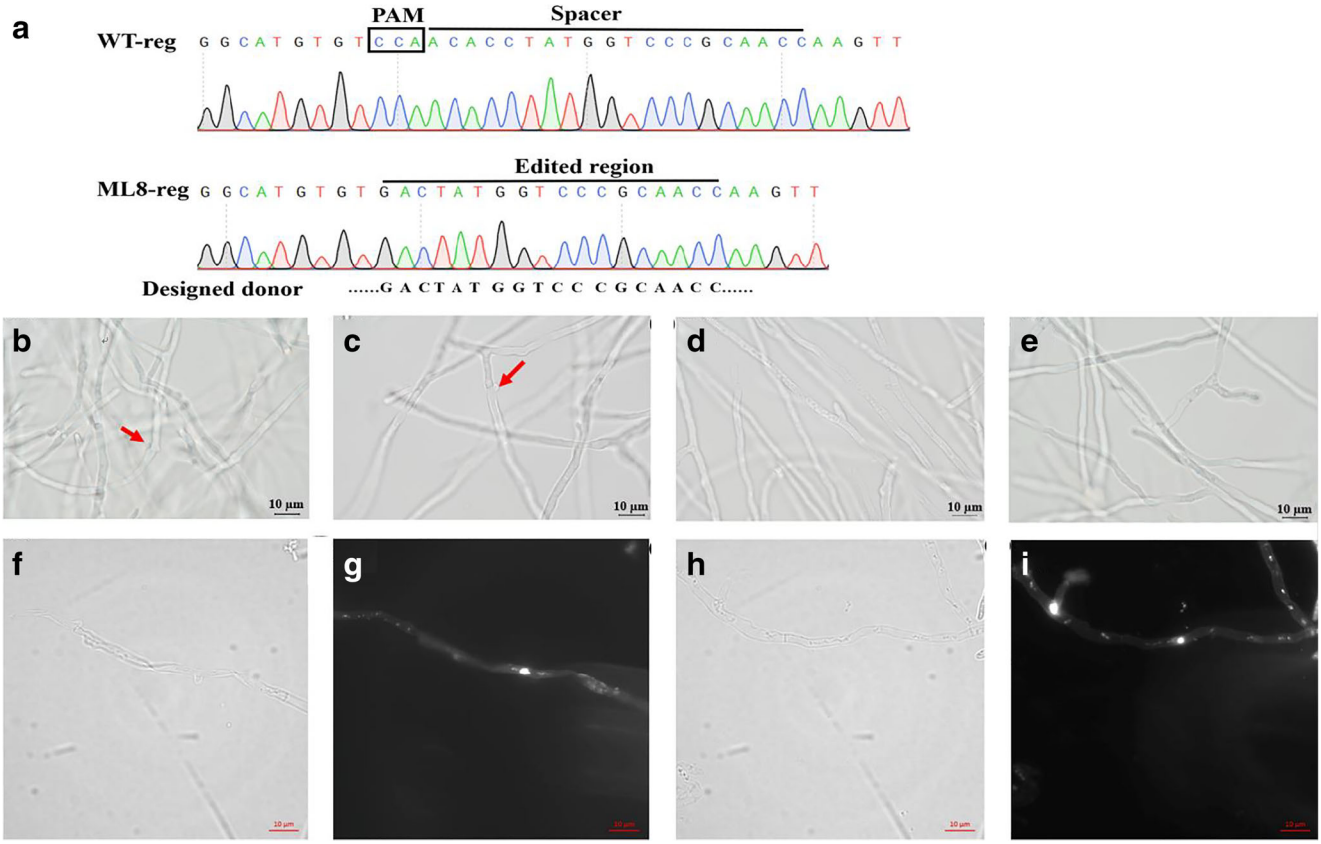

Fig. 4 Monokaryon verified by sequencing confirmation (**a**) and microscopic detection of clamp connection (**b**–**e**) and DAPI staining (**f**–**i**). Black rectangle represents the PAM sequence. The sequence under the blank line was the spacer. The mycelia of WT (**b**, **c**), WT-reg (**d**), *cyp5150l8* mutant ML8-reg (**e**), red arrows represent the clamp connection; the mycelia of WT-reg (**f**, **g**) and ML8-reg (**h**, **i**) observed in bright field and DAPI exciting field respectively

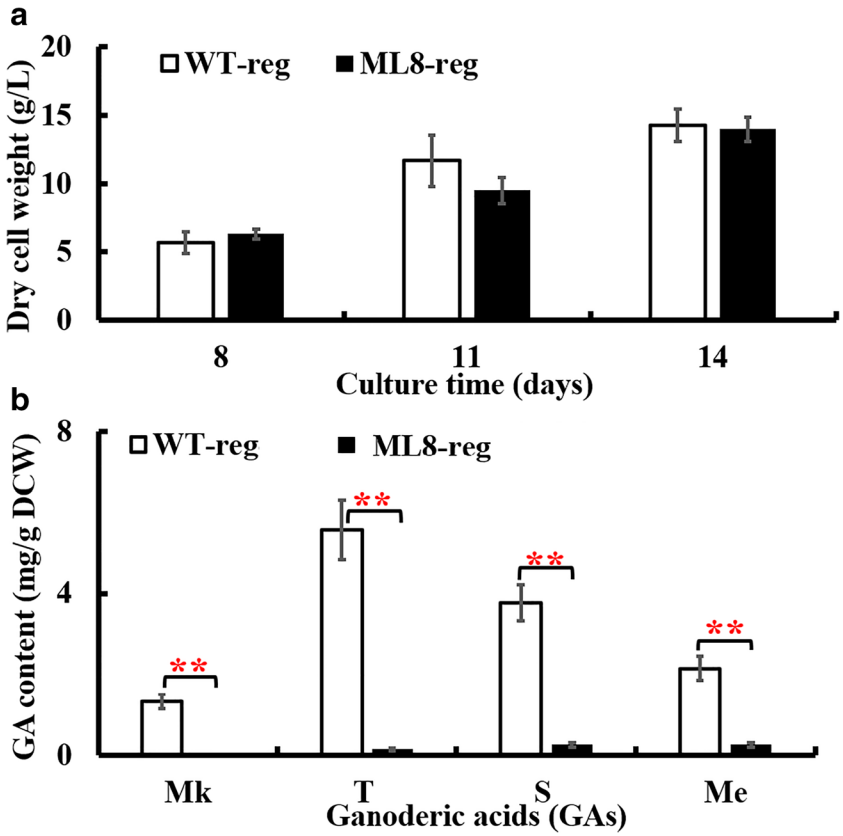

Fig. 5 Cell growth (**a**) and production of ganoderic acids (GAs) at day 11 (**b**) by WT-reg and *cyp5150l8* mutant ML8-reg in liquid static fermentation. WT and the mutant was shown in blank and dark, respectively. The error bars represent standard deviations from three independent samples. $^{**}P < 0.01$

201 bp downstream of the start codon of *cyp505d13* was selected. After transformation and sequence confirmation, two mutants out of nine carboxin-resistant transformants were obtained (Fig. 6), with the editing efficiency of 22.2%. Successful disruption of *cyp505d13* is also a strong evidence supporting that our system is indeed useful for functional gene studies of *G. lucidum*.

Discussion

Functional gene disruption is important but challenging in basidiomycete mushrooms. This is due to (i) the low efficiency of both gene delivery and HR, and (ii) the lack of well-characterized elements for implementing the functionality of genome editing tools for basidiomycete mushrooms.

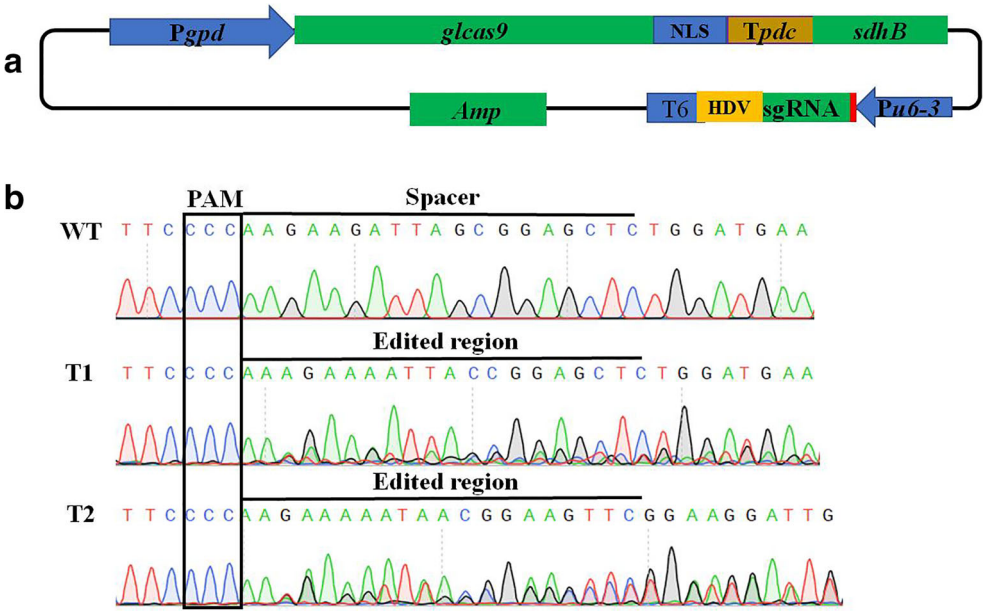

Fig. 6 The editing results of *cyp505d13* mutants. **a** The editing plasmid of *cyp505d13*, p*U6-3-td13*-gRNA-HDV. Red box represents *cyp505d13* spacer (*td13*): GAGCTCCGCTAA TCTTCTT (5′-3′), PAM was "GGG (5′-3′)". **b** Alignment of *cyp505d13* mutants and WT; black rectangle represents the PAM sequence. The sequence under the blank line is the spacer

CRISPR-Cas9 technology provides a possibility to achieve a breakthrough, and here succeeding to our recent work (Qin et al. 2017), the CRISPR-Cas9 system for *G. lucidum* was improved and its gene editing efficiency in *G. lucidum* was enhanced. This system does not require in vitro gRNA preparation and makes one round selection-out of disruptant by using the selective marker from its integrated CRISPR plasmid. In addition, Cas9 and gRNA genes are continuously expressed inside the cell, which may avoid the low delivery efficiency and RNA degradation during transformation of in vitro transcribed gRNA, and eventually contribute to a higher cleavage efficiency by CRISPR-Cas9. Compared to other reports on basidiomycete mushrooms (Table 1), this strategy exhibited a relative high editing efficiency for marker gene, and more importantly, the CRISPR-Cas9 assisted functional gene disruption and precise gene editing in mushroom were successfully achieved.

It is also noted that the *cyp5150l8* disruption efficiency was lower than those of *ura3* and *cyp505d13* (0.67 mutants/10^7 protoplasts vs. 5.3 mutants/10^7 protoplasts and 2 mutants/10^7 protoplasts), which may be attributed to the targeting site dependent effect of CRISPR-Cas9 (Chen et al. 2017; Gao et al. 2017; Jensen et al. 2017). Interestingly, the *cyp5150l8* mutants exhibited a precise repair mode when a long HR donor (2 kb) was adopted. In another medicinal fungus, the ascomycete *Cordyceps militaris* (Chen et al. 2018), a short length of ssDNA HR donor (60 bp), failed to repair the DSB yielded by CRISPR-Cas9. These results indicate donor design may be important to realize CRISPR-Cas9 assisted HR in basidiomycete mushrooms.

For the expression of gRNA in vivo, the results indicated that both p*U6-3* and p*U6-4* enabled expression of gRNA in *G. lucidum*, while p*U6-3* exhibited a higher promoter strength than p*U6-4*. Protoplasts transformed with plasmid p*U6-3-ura3.b*-gRNA were confirmed with a higher expression level of *ura3.b*-gRNA than that transformed with p*U6-4-ura3.b*-gRNA (Fig. 1f), which may lead to the higher editing efficiency for the former. Besides, TATA box was reported critical for the transcription of pol III in eukaryotes (Gao et al. 2018b; Hernandez 2001), but it was not identified in the p*U6-3* sequence of this work. So, why the p*U6-3* had a higher pol III stimulating activity than p*U6-4* in *G. lucidum* remains to be an interesting question, which requires in-depth investigation.

Addition of HDV slightly increased the *ura3* editing efficiency (Fig. 2d and Supplemental Table S2), suggesting the TTTT, a linker used for connection of gRNA and HDV in a previous study (Gao and Zhao 2014), be incomplete for termination of p*U6-3*, and the gRNA structure with a uniform U4 tail may result in a higher editing efficiency compared to gRNA structures with a mixture of poly U tails when targeting at *ura3.b*. However, whether the current gRNA structure with a uniform U4 tail works better when targeting at *ura3.b* or containing a U4 tail in gRNA structure is superior when targeting at different sites is still an open question. Nonetheless, incorporation of HDV or other efficient self-cleavage ribozymes with *u6* promoter for targeting a reporter gene is a convenient way allowing the rapid readout of gRNA working efficiency, which promotes identifying an optimal gRNA design in terms of increasing the editing efficiency of CRISPR-Cas9.

Single allele gene editing was observed in both *ura3* and *cyp5150l8* (data not shown). Since no SNPs were detected in both *ura3* and *cyp5150l8* from the dikaryon *G. lucidum* (Figs. S2A, B), a possible explanation is that the CRISPR-Cas9 was only targeted to one nucleus of the dikaryon strain. To solve this problem, isolation of homozygous mutants was done as described and succeeded here. In addition, adoption of a monokaryon strain or germinating spore for transformation may be also feasible (Kim et al. 2015).

The C-26 oxidation of lanosterol by CYP5150L8 was proposed as the initial step for GA biosynthesis from our recent synthetic biology work (Wang et al. 2018), and its three-step catalytic products, i.e., HLDO, HLDA and HLDOA, which were considered as precursors for biosynthesis of other GAs could be detected neither in WT nor in *cyp5150l8* disrupted strain, suggesting a fast conversion of these products by downstream enzymes. Nevertheless, significantly reduced titers of four GAs (GA-Mk, T, S, Me) were observed in *cyp5150l8* disruptant compared to WT (Fig. 5b). The results were in a good agreement with our previous hypothesis that this CYP gene was critical to the overall pathway of GA biosynthesis from lanosterol in *G. lucidum* (Wang et al.

Table 1 Summary of CRISPR-Cas9 based gene editing in basidiomycete mushrooms as reported

Mushroom	Expression of gRNA	Target gene	Editing efficiency (mutants/10^7 protoplasts)	Total mutants	Repair mode	Reference
Ganoderma lucidum	In vitro	*ura3*	0.2–1.78	4	NHEJ	Qin et al. (2017)
Coprinopsis cinerea	In vivo	*egfp*	0.2	2	NHEJ	Sugano et al. (2017)
Schizophyllum commune	In vitro	*hom2*	0.15–1.8	17	HR	Vonk et al. (2019)
G. lucidum	In vivo	*ura3*	5.3	54	NHEJ	This work
G. lucidum	In vivo	*cyp5150l8*	0.67	2	HR	This work
G. lucidum	In vivo	*cyp505d13*	2	2	NHEJ	This work

2018). It should be also noted that four GAs can still be detected in *cyp5150l8* disruptant, suggesting that another pathway from lanosterol to GAs bypassing CYP5150L8 may exist, and this point may be very interesting to the studies on GA biosynthetic pathway and related functional genes and enzymes.

In conclusion, with the assistance of CRISPR-Cas9 capable of in vivo gRNA expression, a functional gene *cyp5150l8* in the traditional medicinal mushroom *G. lucidum* has been successfully disrupted. On one hand, it could serve as a vital platform for metabolic engineering of *G. lucidum* and provides lessons to genetic engineering of other basidiomycete mushrooms, as reported in industrial microorganisms (Jiang et al. 2017) and mammalian production systems (Lee et al. 2015). On the other hand, it will enable in situ biosynthetic mechanism studies and accelerate our understanding towards this important organism. The developed tool and related information would also be valuable to studies on other basidiomycete mushrooms.

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Compliance with ethical standards

Conflict of interest JJZ, PAW, and HX are the co-inventors of a functional gene editing technology in Lingzhi, which is covered under a Chinese patent (Application No. 201811522445.0) with a priority date Dec. 13, 2018.

Ethical approval This article does not contain any studies with human participants or animals experiments.

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