RESEARCH ARTICLE

Efficient CRISPR/Cas9 system based on autonomously replicating plasmid with an AMA1 sequence and precisely targeted gene deletion in the edible fungus, *Cordyceps militaris*

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Abstract

Cordyceps militaris is a popular edible fungus with important economic value worldwide. In this study, an efficient CRISPR/Cas9 genome-editing system based on an autonomously replicating plasmid with an AMA1 sequence was constructed. Further, a precisely targeted gene deletion via homology-directed repair was effectively introduced in *C. militaris*. Gene editing was successful, with efficiencies of 55.1% and 89% for *Cmwc-1* and *Cmvvd*, respectively. Precisely targeted gene deletion was achieved at an efficiency of 73.9% by a single guide RNA supplementation with donor DNAs. Double genes, *Cmwc-1* and *Cmvvd*, were edited simultaneously with an efficiency of 10%. Plasmid loss was observed under non-selective culture conditions, which could permit recycling of the selectable marker and avoid the adverse effects of the CRISPR/Cas9 system on the fungus, which is beneficial for the generation of new cultivars. RNA Pol III promoters, endogenous $tRNA_{Pro}$ of *C. militaris,* and chimeric $AfU6$-$tRNA_{Gly}$ can be used to improve the efficiency. Polyethylene glycol-mediated protoplast transformation was markedly more efficient than *Agrobacterium tumefaciens*-mediated transformation of *C. militaris*. To our knowledge, this is the first description of genome editing and precisely targeted gene deletion in mushrooms based on AMA1 plasmids. Our findings will enable the modification of multiple genes in both functional genomics research and strain breeding.

Guoliang Meng and Xuping Wang authors contributed equally to this work.

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INTRODUCTION

Cordyceps militaris (L.) Fr., which belongs to ascomycetes, is a well-known edible and medicinal fungus that has been widely used as an herbal tonic in East Asia. Owing to the successful cultivation and commercialization of fruiting bodies, this fungus has become a valuable mushroom with important economic value worldwide. *C. militaris* produces a variety of bioactive components, such as cordycepin (Cunningham et al., 1950), pentostatin (Xia et al., 2017), N^6-(2-hydroxyethyl)-adenosine (Zhang et al., 2018), carotenoids (Fu, 2005), ergosterol (Nallathamby et al., 2015) and polysaccharides (Gai & Zhang, 1992).

The biosynthetic pathways of bioactive components and increasing the contents of these components via genetic engineering have been the focus of research on this fungus (Lou et al., 2019; Xia et al., 2017). Gene disruption, overexpression and complementation can be achieved by homologous recombination via *Agrobacterium tumefaciens*-mediated transformation (ATMT) in this fungus (Zheng et al., 2011; Yang et al., 2016; Zhang et al., 2020). However, the low efficiency of gene manipulation mediated by homologous recombination has markedly hindered high-throughput gene function studies.

Strain degeneration occurs at high frequency during the subculture and preservation of *C. militaris*, which hinders development of the industry (Lou et al., 2019; Sun et al., 2017). Therefore, strain improvement and breeding of this fungus are important. Traditional breeding is untargeted, laborious and time-consuming. Molecular breeding can remarkably improve breeding efficiency. However, such strains are classified as genetically modified organisms owing to the introduction of foreign DNA sequences, especially antibiotic resistance genes (Wohlers, 2015). In 2016, *Agaricus bisporus* with a CRISPR/Cas9-edited genome was reported to escape the supervision of the US Department of Agriculture (Waltz, 2016). Now CRISPR-enabled engineering is being used in commercial and model crops to increase yield, improve drought tolerance and increase growth in limited-nutrient conditions, and breed crops with improved nutritional properties (Barrangou & Doudna, 2016).

The CRISPR/Cas9 system has since been used for gene editing in higher fungi, including *Coprinopsis cinerea* (Sugano et al., 2017), *Ganoderma lucidum* and *Ganoderma lingzhi* (Qin et al., 2017; Wang et al., 2020), *Schizophyllum commune* (Jan Vonk et al., 2019), *Pleurotus ostreatus* (Boontawon et al., 2021), and *Pleurotus eryngii* (Wang et al., 2021). In *C. cinerea*, strong promoters of *CcDED1* and *U6-snRNA* were used to express Cas9 and sgRNA, respectively, and CRISPR/Cas9-mediated *GFP* mutagenesis was successful in a stable *GFP* expressing strain with an efficiency of 10.5% (Sugano et al., 2017). The *ura3* gene was successfully edited by codon-optimized Cas9 and in vitro-transcribed sgRNA in *G. lucidum* and *G. lingzhi* (Qin et al., 2017). *cyp5150l8*, a cytochrome P450 monooxygenase (CYP450) gene responsible for the biosynthesis of ganoderic acids, was edited in *G. lucidum* (Wang et al., 2020). In *Schizophyllum commune*, a homeodomain transcription factor gene, *hom2*, was successfully edited using pre-assembled Cas9 ribonucleoproteins (RNPs) (Jan Vonk et al., 2019). Plasmids containing the Cas9 expression cassette and sgRNAs for *fcy1* and *pyrG* were transferred to protoplasts of *Pleurotus ostreatus*, resulting in gene-editing efficiencies of 20–94.9% (Boontawon et al., 2021). A highly efficient *pyrG* gene editing system was established in *P. eryngii* using an in vivo-synthesized *U6* promoter-guided sgRNA and codon-optimized Cas9 (Wang et al., 2021).

In *C. militaris*, the *pyrG* gene was successfully edited using the CRISPR/Cas9 system; however, the efficiency was only 11.76% (Chen et al., 2018). The editing efficiency of *Cmura5* reached 100% when an optimized ribonucleoprotein (RNP)-based method was employed (Zou et al., 2021). These studies indicate the feasibility of gene editing in this species. However, the *ura* gene was selected as a target and the editing efficiency was based on screening on media containing 5-fluoro orotic acid. To date, multiple gene editing has seldom been reported in this species.

Plasmids containing the AMA1 sequence from *Aspergillus nidulans* Glasgow strains can perform extrachromosomal replication (Gems et al., 1991). AMA1 plasmids have a low potential of insertion into the fungal genome during transformation and can be easily lost after being cultured under nonselective conditions, allowing the reuse of dominant selection marker(s) in the next transformation (Wang & Coleman, 2019).

In theory, the AMA1-based CRISPR/Cas9 genome editing technology enables limitless rounds of genetic engineering. Therefore, AMA1 plasmids have been used in the CRISPR/Cas9 systems for filamentous fungi (Wang & Coleman, 2019). For example, an efficient multiple gene modification was constructed using the CRISPR/Cas9 system based on AMA1 in *Aspergillus oryzae* (Katayama et al., 2019). Nonetheless, no reports have revealed whether AMA1-based plasmids can function and be recycled in the CRISPR/Cas9 systems for ascomycetous mushrooms.

The CRISPR/Cas9 system has revolutionized genetic manipulation; however, precisely and efficiently deleting genes remains a challenge (Li & Huang, 2021). Double-stranded breaks (DSBs) at the target site can be introduced by the Cas9 protein recruited by single-guide RNAs, which trigger DNA repair mediated by non-homologous end-joining (NHEJ), resulting in targeted insertions or deletions (indels) (Hsu et al., 2014). Although the process is efficient, indels are unpredictable, imprecise, and

typically very small in size (Choi et al., 2021). Precise gene manipulation is critical for exploring gene functions.

In this study, an AMA1-based CRISPR/Cas9 system was successfully constructed in *C. militaris*. Double-gene editing was also successful, with an efficiency of 10%. The system was further optimized by comparing different RNA polymerase III (RNA Pol III) promoters. Endogenous $tRNA_{Pro}$ of *C. militaris* and the heterologous chimeric RNA Pol III promoter $AfU6\text{-}tRNA_{Gly}$ can improve efficiency. Plasmid loss under non-selective culture conditions allows the reuse of markers for subsequent transformation procedures. Precisely targeted gene deletion, based on homology-directed repair (HDR) templates, was performed. Finally, using the same plasmid, the efficiency of PEG-mediated protoplast transformation and ATMT in this species was compared.

EXPERIMENTAL PROCEDURES

Strains and cultural conditions

The *C. militaris* strain, CGMCC 3.16323, was maintained on potato dextrose agar (PDA) at 20°C. The *Escherichia coli* strain, DH5α, and *A. tumefaciens* strain, AGL-1, which were used for the construction of recombinant plasmids, were purchased from Tiangen Biotech Co., Ltd. (Beijing, China) and stored at –80°C. *E. coli* strains were cultured in Luria-Bertani medium supplemented with kanamycin (50 μg/ml) as a selection marker. *A. tumefaciens* strains were cultured in yeast extract beef (YEB) medium supplemented with carbenicillin (50 μg/ml) and kanamycin (50 μg/ml).

Plasmid construction

The Cas9-expressing plasmids, pAMA1-Cas9-hygR, pLC1, and pLC3 (Chen et al., 2020), conferring hygromycin resistance, were generously gifted by Professor Gang Liu from the Institute of Microbiology, Chinese Academy of Sciences. These plasmids contained the same Cas9-expressed cassettes, in which the coding sequences of *cas9* from *Streptococcus pyogenes* with a 3×Flag tag, an SV40 nuclear location signal (NLS) in its 5′ terminus and a nucleoplasm NLS in its 3′ terminus were placed under the control of the *gpd* promoter and *trpC* terminator from *A. nidulans* (Figure 1A). An autonomously replicating sequence, AMA1, from filamentous fungi (Zhang et al., 2016) was presented in pAMA1-Cas9-hygR (Figure 1A). pLC1 and pLC3 were the Ti plasmids. All plasmids and primers used in this study are listed in Tables S2 and S3, respectively.

To evaluate the efficiency of the CRISPR/Cas9 system in *C. militaris*, the photoreceptor genes, *Cmwc-1*

and *Cmvvd*, were chosen as target sites. The *Cmwc-1* gene-editing plasmid, pAMA1-Cas9-sgRNA$_{Cmwc-1}$, was constructed as follows: *Neurospora crassa 5SrRNA* (E00012) downloaded from the *5SrRNA* database (http://combio.pl/rrna/) was selected as the promoter for sgRNA. A 20 bp guide sequence (crRNA$_{Cmwc-1}$, cacgctccaacgcattcga) targeting *Cmwc-1* was designed using the eukaryotic pathogen CRISPR guide RNA/DNA Design Tool (http://grna.ctegd.uga.edu/). The entire sgRNA$_{Cmwc-1}$ sequence (Nc5SrRNA-crRNA$_{Cmwc-1}$-tracrRNA-T$_6$) was synthesized by Sangon Biotechnology Company (Shanghai, China) and inserted into pAMA1-Cas9-hygR at the *Not*I site (Figure 1A) using a CloneExpress® Ultra One Step Cloning Kit (Vazyme Biotech Co., Ltd, Beijing, China) to obtain pAMA1-Cas9-sgRNA$_{Cmwc-1}$. The *Cmvvd*-editing plasmid, pAMA1-Cas9-sgRNA$_{Cmvvd}$, was almost identical to pAMA1-Cas9-sgRNA$_{Cmwc-1}$, except for the 20 bp guide sequence that targeted *Cmvvd* (crRNA$_{Cmvvd}$, gcggatccaactgttcaact) (Figure S1A).

A plasmid for targeted gene deletion was constructed using a similar strategy. To delete the 2.963 kb DNA fragment of *Cmwc-1*, pAMA1-Cas9-sgRNA$_{Cmwc-1}$ was used. The HDR templates (donor DNAs) of the targeted loci were amplified from *C. militaris* using primers, CMwc1up-F/R and CMwc1down-F/R. The templates were then cloned into pAMA1-Cas9-sgRNA$_{Cmwc-1}$ to construct the plasmid, pAMA1-Cas9-sgRNA$_{Cmwc-1-HR}$ (Figure 2A).

The double-gene-editing plasmid, pAMA1-Cas9-sgRNA$_{Cmwc-1/Cmvvd}$ (Figure 3A), for *Cmwc-1* and *Cmvvd* was constructed by inserting cassettes Nc5SrRNA-crRNA$_{Cmwc-1}$-tracrRNA-T$_6$ and Nc5SrRNA-crRNA$_{Cmvvd}$-tracrRNA-T$_6$ into the pAMA1-Cas9-hygR plasmid at the *Not*I loci.

Optimization of the RNA pol III promoters for gRNA expression

The RNA Pol III promoters for gRNA expression were optimized. Endogenous tRNAs of *C. militaris* were identified using the tRNAscan-SE website (http://lowelab.ucsc.edu/tRNAscan-SE/) with the genome of the *C. militaris* strain CM01 (Zheng et al., 2011). Based on the efficiency of $tRNA_{Pro}$ and $tRNA_{Glu}$ in the CRISPR/Cas9 system (Mefferd et al., 2015), $tRNA_{Pro}$ and $tRNA_{Glu}$ in *C. militaris* were selected from a variety of predicted tRNAs. To confirm the transcription initiation of sgRNAs, 94 bp, and 80 bp upstream of $tRNA_{Pro}$ and $tRNA_{Glu}$, respectively, were also truncated with these two tRNAs as the RNA Pol III promoters. The heterologous chimeric RNA Pol III promoter, $AfU6\text{-}tRNA_{Gly}$, was amplified from pLC3 (Chen et al., 2020) and used as an RNA Pol III promoter. Plasmids with sgRNA targeting *Cmwc-1* under the control of these three RNA Pol III promoters were constructed
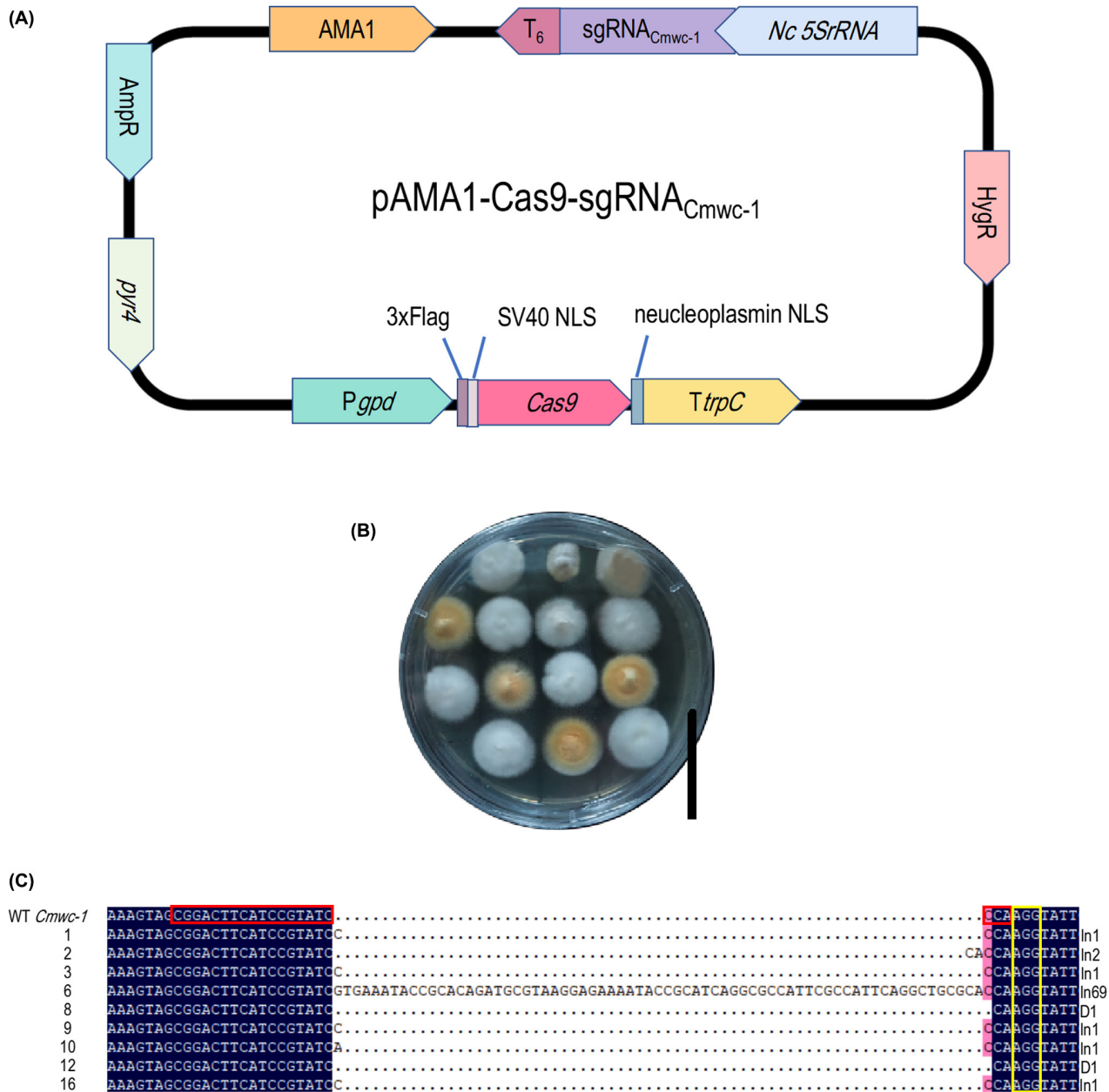
FIGURE 1 *Cmwc-1* was edited by an AMA1-based CRISPR/Cas9 system via PEG-mediated protoplast transformation. (A) Schematic of the pAMA1-Cas9-sgRNA$_{Cmwc-1}$ plasmid expressing Cas9 and sgRNA$_{Cmwc-1}$. (B) Colony of the pAMA1-Cas9-sgRNA$_{Cmwc-1}$ protoplast transformants on selective medium after light exposure. Bar = 3 cm. (C) DNA sequences of the white colony of pAMA1-Cas9-sgRNA$_{Cmwc-1}$ protoplast transformants at the *Cmwc-1* target site. 1, 2, 3, 6, 8, 9, 10, 12 and 16 represent the white colonies; WT indicates the wild-type strain. In1, In2 and In69 represent 1 bp, 2 bp and 69 bp insertions, respectively; D1 represents a 1 bp deletion. The yellow and red boxes indicate the PAM and guide sequence, respectively.

using the same strategy: tRNA$_{Pro}$-crRNA$_{Cmwc-1}$-tracrRNA-T$_6$, tRNA$_{Glu}$-crRNA$_{Cmwc-1}$-tracrRNA-T$_6$ and AfU6-tRNA$_{Gly}$-crRNA$_{Cmwc-1}$-tRNA$_{Gly}$-T$_6$ were integrated into pAMA1-Cas9-hygR at the *Not*I loci using a CloneExpress Ultra One Step Cloning Kit. These plasmids were transformed into *C. militaris* by PEG-mediated protoplast transformation, and *Cmwc-1*-edited strains were confirmed by sequencing. Three biological repeats were performed for each RNA Pol III promoter.

PEG-mediated protoplast transformation

Protoplast preparation and PEG-mediated transformation were performed according to a previously described procedure (Lou et al., 2018) with some modifications. The *C. militaris* strain was cultured in 100 ml of PPDB (potato dextrose broth supplied with 1% peptone) in a static state at 20°C for 4 days. Fresh mycelia were collected via filtering with four layers of lens paper and successive cleaning with adequate ddH$_2$O and 0.8 mol/L KCl.
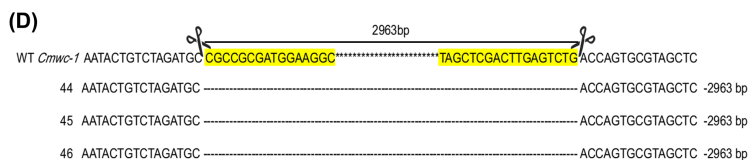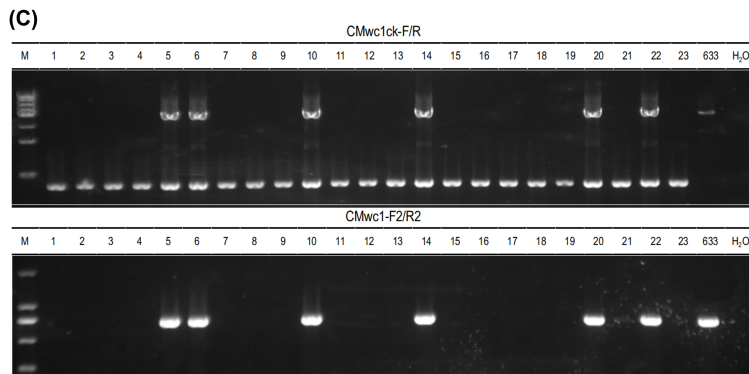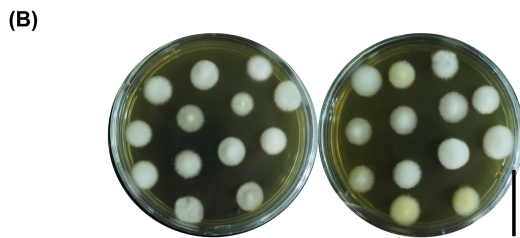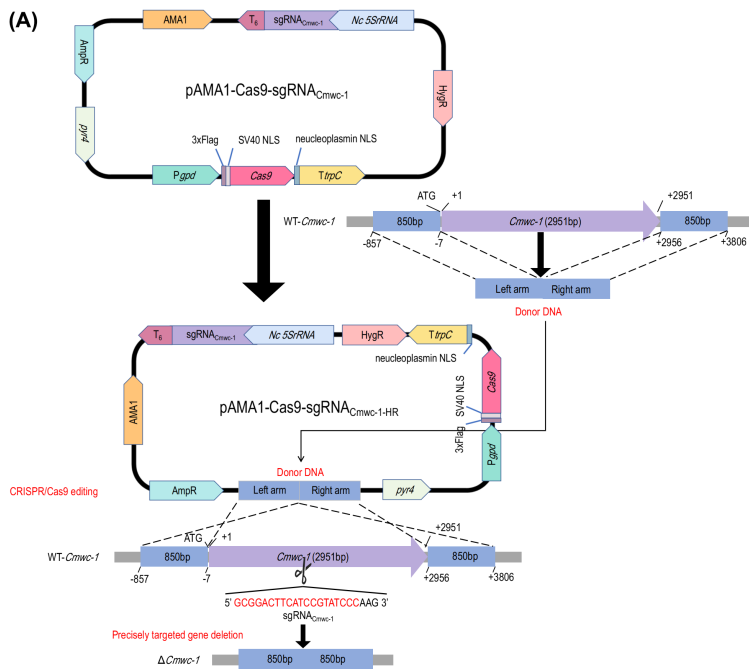
FIGURE 2 Precisely targeted gene deletion of *Cmwc-1* by the AMA1-based CRISPR/Cas9 system supplemented with donor DNAs. (A) The strategy of targeted gene deletion of *Cmwc-1*. 850-bp DNA fragments for the left and right arms flanking the target gene of *Cmwc-1* were cloned as donor DNAs. Thereafter, the donor DNAs were cloned into pAMA1-Cas9-sgRNA$_{Cmwc-1}$ to construct the pAMA1-Cas9-sgRNA$_{Cmwc-1-HR}$ plasmid. The target gene of *Cmwc-1* was deleted precisely by a single guide RNA supplementation with donor DNAs in *C. militaris*. (B) Colony of the pAMA1-Cas9-sgRNA$_{Cmwc-1-HR}$ protoplast transformants on selective medium after light exposure. Bar = 3 cm. (C) Verification of the *Cmwc-1* deletion strain by PCR. 1–23 are transformants, 633 is the wild-type strain, H$_2$O represents the blank controls. (D) DNA sequences of the target fragment of the *Cmwc-1* deletion strains. WT indicates the wild-type strain. * indicates the omitted fragments, –- indicates deleted fragments.

To generate protoplasts, 0.2 g of wet mycelia were digested in 1 ml of 2% lywallzyme (dissolved with 0.8 mol/L KCl, pH = 6.5, Guangdong Culture Collection Center, Guangzhou, China) at 32°C with gentle shaking (90 rpm) for 3 h, and the formed protoplasts were filtered with four layers of lens papers, washed twice with 0.8 mol/L KCl by centrifugation at 3000 rpm for 10 min at room temperature, and then suspended in 100–300 μl of STC buffer (1 M sorbitol, 10 mM Tris–HCl, 25 mM CaCl$_2$, pH = 7.5) to a final density of 3×10^8 cells/ml for use.

Plasmid transformation was subsequently performed. Briefly, for each 100 μl of protoplast, 2 μg of plasmid was added. After incubation on ice for 5 min, 50 μl of PEG buffer (25% PEG, 10 mM of Tris–HCl, and 25 mM of CaCl$_2$, pH = 7.5) was slowly added and placed on ice for 30 min. Thereafter, 0.5 ml PEG buffer was slowly added

FIGURE 3 Double gene editing for genes, *Cmwc-1* and *Cmvvd*, by the AMA1-based CRISPR/Cas9 system. (A) Schematic of the double-gene-editing plasmid. Cassettes Nc5SrRNA-sgRNA $_{Cmwc-1}$-T6 and Nc5SrRNA-sgRNA $_{Cmvvd}$-T6 were inserted into the pAMA1-Cas9-hygR plasmid. (B) Colony of pAMA1-Cas9-sgRNA$_{Cmwc-1/Cmvvd}$ transformants on selective medium under dark conditions and after light exposure. Bar = 3 cm. (C) DNA sequences of the randomly selected transformants at the *Cmwc-1* target site. (D) DNA sequences of the randomly selected transformants at the *Cmvvd-1* target site. 3, 4, 6, 7, 8, 9, 10, 13, 14 and 15 represent the selected colonies, WT indicates the wild-type strain, and In1 represents a 1 bp insertion. Yellow and red boxes indicate PAM and guide sequences, respectively.
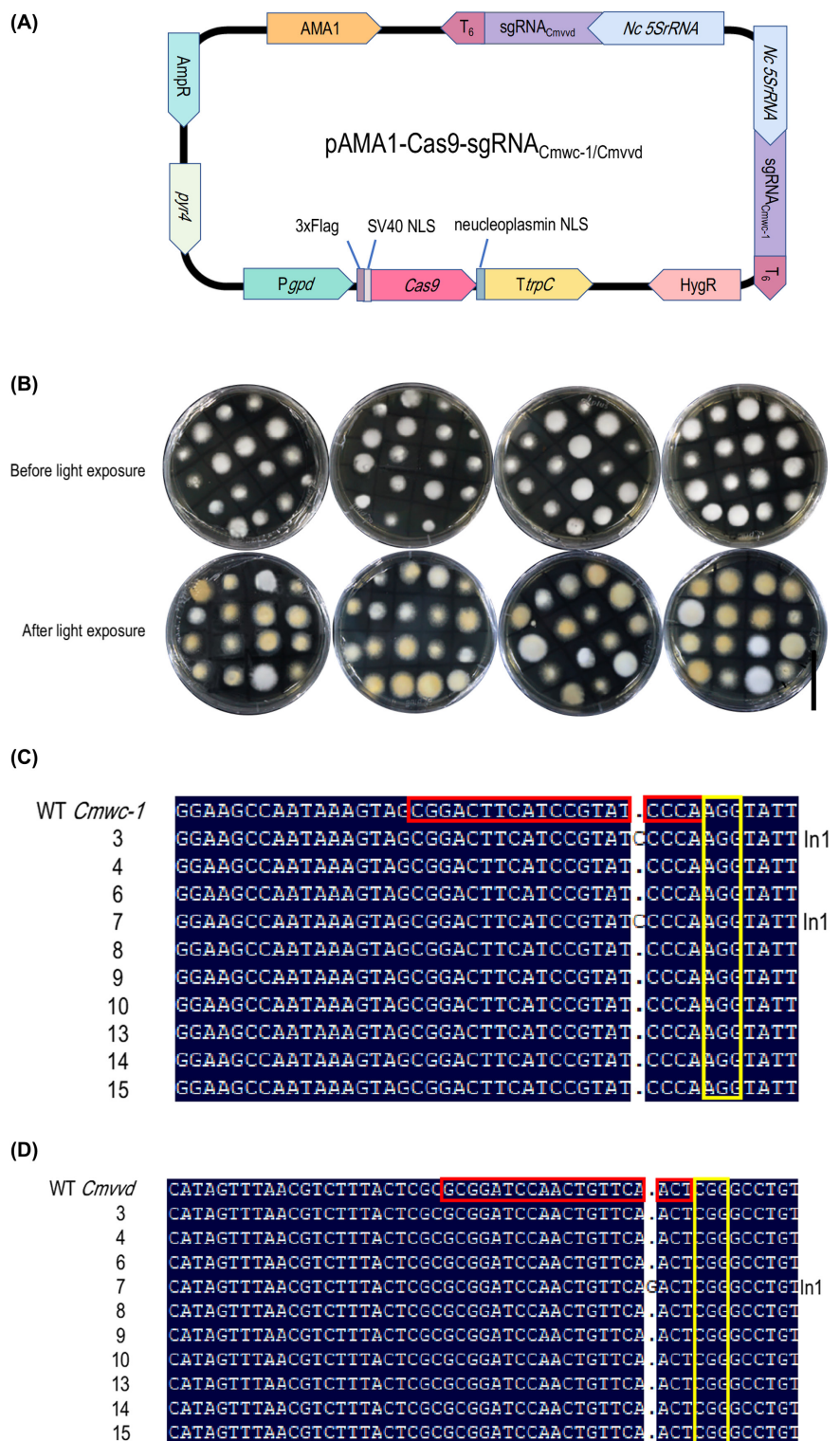
and incubated at 28°C for 20 min, and then 1 ml STC buffer was slowly added. Cells were harvested by centrifugation at 3000 rpm for 10 min at room temperature, resuspended in 200–400 μl of STC buffer and incubated in PPDA with 1 M mannitol and 500 μg/ml hygromycin at 25°C. After 4–7 days of regeneration, individual colonies germinated visibly and were transferred to another selection plate (PPDA+500 μg/ml hygromycin).

Selection of the edited and targeted gene deletion strains

To identify the *Cmwc-1*-edited strains, pAMA1-Cas9-sgRNA$_{Cmwc-1}$ transformants were cultured under a 12 h light/12 h dark cycle for three days, and the colour of the colonies was observed. White colonies were selected, and PCR analysis of the targeted loci of *Cmwc-1* was

performed with Cmwc1-F1/R1 primers followed by sequencing. For the targeted gene deletion strains, PCR analysis of the targeted fragment of *Cmwc-1* was performed using the primers, Cmwc1ck-F/R, and three randomly selected strains were sequenced. For the *Cmvvd*-edited strains, nine randomly selected strains were sequenced around the target site using primers, Cmvvdck-F/R. For the double-gene-edited strains, primers Cmvvdck-F/R and Cmwc1-F1/R1 were used to sequence 9 randomly selected strains.

Loss of the AMA1 plasmid

Plasmids with AMA1 autonomously replicating sequences could exist independently of genomic DNA and may be lost in daughter cells during the cell division process under non-selective culture conditions. Plasmid loss was assessed by picking the cells with toothpicks and performing subculture. The *Cmwc-1*-edited strains were cultured on PPDA plates at 20°C for 20 days. The cells were then picked using a sterile toothpick and transferred to new PPDA plates with or without 500 μg/ml hygromycin. Growth was observed after 5–7 days of cultivation. This step was repeated for three generations, and plasmid loss was subsequently identified by *hyg* and ITS4/5 PCR amplification. ITS4/5 was used as a positive control to ensure the quality of the exact DNA and *hyg* was amplified to assess the feasibility of plasmid loss.

Efficiency comparison between ATMT and PEG-mediated protoplast transformation

A Ti plasmid pLC1-sgRNA$_{Cmwc-1}$ targeting the *Cmwc-1* gene under the efficient *AfU6-tRNA$_{Gly}$* RNA Pol III promoter was constructed by inserting AfU6-tRNA$_{Gly}$-crRNA$_{Cmwc-1}$-tRNA$_{Gly}$-T$_6$ into pLC1 at the *Sbf*I loci with a CloneExpress® Ultra One Step Cloning Kit. pLC1-sgRNA$_{AfU6-tRNAGly}$ was transformed into *C. militaris* by ATMT, as previously described (Yang et al., 2016), and by PEG-mediated protoplast transformation. The *Cmwc-1*-edited strains were confirmed by sequencing the white colonies at the target loci using primers Cmwc1-F/R.

RESULTS

Editing of Cmwc-1 or Cmvvd using the AMA1-based CRISPR/Cas9 system

The photoreceptor genes, *Cmwc-1* and *Cmvvd*, were selected as the target sites as the phenotypes of albinism or the deepened colour of mycelia were confirmed when *Cmwc-1* and *Cmvvd* were disrupted, respectively,

by homologous recombination via ATMT in our previous studies (Yang et al., 2016; Zhang et al., 2020). After the introduction of pAMA1-Cas9-sgRNA$_{Cmwc-1}$ into the protoplasts of *C. militaris*, 14–24 transformants for three batches were obtained and transferred to light conditions (600–800 lux, 12 h light/12 h dark). More than half of transformants showed pure white colonies (Figure 1B), identical to the albinism phenotype of *Cmwc-1*-disrupted strains obtained by homologous recombination-mediated gene knockout (Yang et al., 2016). PCR and sequencing confirmed that all albinism transformants had been edited at the *Cmwc-1* target site (Figure 1C) around the PAM sequence (AGG), the typical target site of the CRISPR/Cas9 system, whereas none of the orange colonies had a sequence change around the target site, with an actual editing efficiency of 55.1 ± 3.66% with three repeats (Table S1).

For the other blue light receptor-coding gene, *Cmvvd*, more than 80 colonies germinated after protoplast transformation of pAMA1-Cas9-sgRNA$_{Cmvvd}$. To calculate the editing efficiency without phenotype selection, nine transformants were randomly selected and sequenced. Eight of the transformants were identified to be edited at the target site, with an efficiency of 88.89% (Figure S1).

CRISPR/Cas9-mediated precisely targeted gene deletion following supplementation with donor DNAs

The gene was successfully edited using the AMA1-based CRISPR/Cas9 system. However, the modification at the target loci was random and unpredictable, and mutagenesis observed in the targeted transformants was mainly 1–3 bases of deletion/insertion near the PAM sites (Figure 1 and Figure S1). Precise manipulation of genomic sequences can enable investigations on the functions of specific genes and is useful for genetic analysis in basic studies as the genotype (mutant or WT) can be easily determined by conventional genomic PCR experiments. Herein, 850 bp DNA fragments for the left and right arms flanking the target gene of *Cmwc-1* were cloned and used as HDR templates (Figure 2A). The pAMA1-Cas9-sgRNA$_{Cmwc-1-HR}$ plasmid was constructed by inserting an HDR template into the pAMA1-Cas9-sgRNA$_{Cmwc-1}$ plasmid. After pAMA1-Cas9-sgRNA$_{Cmwc-1-HR}$ was introduced, 24 colonies were randomly selected and analyzed by PCR using the primers, CMwc1ck-F/R. A total of 17 colonies (73.9%) showed the expected band at 847 bp (Figure 2B, C). Sequencing analysis revealed that the 2.963 kb DNA fragment, which is the coding region of the *Cmwc-1* gene, was successfully deleted (Figure 2D). These results indicate that the improved CRISPR/Cas9 system is efficient for precisely targeted gene deletion in *C. militaris*.

Simultaneously targeted mutagenesis of Cmwc-1 and Cmvvd using the CRISPR/Cas9 system

Simultaneous mutagenesis of two or more genes is essential for identifying biological pathways and conducting research on gene family. We proceeded to simultaneously edit *Cmwc-1* and *Cmvvd*. The *Cmwc-1*/*Cmvvd*-editing plasmid, pAMA1-Cas9-sgRNA$_{Cmwc-1/Cmvvd}$, was constructed by integrating sgRNA$_{Cmwc-1}$ and sgRNA$_{Cmvvd}$ into pAMA1-Cas9-hygR (Figure 3A). This plasmid was then transformed into *C. militaris* by PEG-mediated transformation. Ten transformants were randomly selected and sequenced (Figure 3B). However, only one colony was confirmed to be edited at both target sites; the other transformants were edited only at the *Cmwc-1* or *Cmvvd* sites (Figure 3C, D).

Optimization of the CRISPR/Cas9 system based on a comparison of gene editing efficiency with different RNA pol III promoters

As the double-gene-editing efficiency of *Cmwc-1*/*Cmvvd* using this CRISPR/Cas9 system was only 10%, the CRISPR/Cas9 system was further optimized by comparing the *Cmwc-1*-editing efficiency with different RNA Pol III promoters. Endogenous tRNAs of *C. militaris*, *tRNA$_{Pro}$*, and *tRNA$_{Glu}$*, and the heterologous chimeric RNA Pol III promoter, *AfU6-tRNA$_{Gly}$*, from *A. fumigatus* Af293.1 (Chen et al., 2020) were used (Figure S2A). As shown in Figure S2B, transformants appeared as white colonies after light exposure, resembling the *Cmwc-1* disruption phenotype. Nine white colonies of each transformant type were randomly selected and sequenced at the target loci. The overwhelming majority of selected white transformants were edited in the *Cmwc-1* gene, whereas only seven of the nine selected pAMA1-Cas9-sgRNA$_{tRNA-Glu}$ white transformants were edited (Figure S2C). After three repetitions of the experiment, the editing efficiencies of pAMA1-Cas9-sgRNA$_{tRNA-Pro}$, pAMA1-Cas9-sgRNA$_{tRNA-Glu}$ and pAMA1-Cas9-sgRNA$_{AfU6-tRNA}$ were 84.7%, 59.5%, and 77.5%, respectively, significantly higher than that of pAMA1-Cas9-sgRNA$_{Cmwc-1}$ (*Nc-5SrRNA*) 55.1% ($p < 0.05$), indicating successful optimization of the RNA Pol III promoters (Figure 4).

Plasmid loss in the edited strains under nonselective culture conditions

To reduce the off-target potential owing to the long existence of Cas9 and sgRNA and preparation for further gene complementation in edited strains, the reuse of
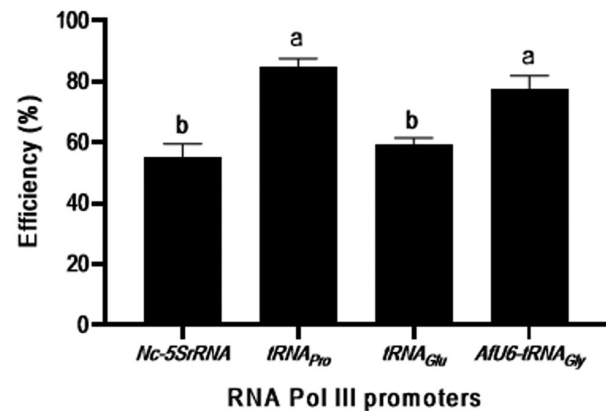
FIGURE 4 The editing efficiency with different RNA pol III promoters. Different letters above the bar indicate the significant difference (one-way ANOVA, $p < 0.05$).

Cas9, sgRNA, and selection markers was considered in succession after the successful adaptation and optimization of the CRISPR/Cas9 system in *C. militaris*. As described previously, the autonomously replicated sequence, AMA1, could enable plasmid loss during cell division by repeated culturing on nonselective media (Nødvig et al., 2015; Weyda et al., 2017). In this study, plasmid loss was achieved by picking cells with toothpicks and culturing them on agar plates. After three generations of culture, no colonies had formed on the selective agar plates after 10 days; however, mycelia could be clearly observed on non-selective agar plates (Figure S3A), indicating that plasmids with the AMA1 sequence were lost during cell reproduction.

To determine whether plasmids were lost in colonies that germinated on agar medium, *hyg* and ITS4/5 sequences were amplified when the colonies were large enough. All nine randomly selected single colonies on the non-selective media had clear gel electrophoresis bands of ITS 4/5, indicating good DNA quality. However, no *hyg* sequence was amplified, indicating the loss of the pAMA1-Cas9-sgRNA$_{Cmwc-1}$ plasmid (Figure S3B).

PEG-mediated protoplast transformation has a remarkably higher efficiency than ATMT

A. tumefaciens-mediated transformation (ATMT) has been widely used for gene deletion, complementation, and overexpression in *C. militaris* (Xia et al., 2017; Zhang et al., 2020; Zheng et al., 2011). The same Ti plasmid, pLC1-sgRNA$_{Cmwc-1}$, targeting *Cmwc-1* under the efficient promoter of *AfU6-tRNA$_{Gly}$* was transformed by PEG-mediated protoplast transformation and ATMT, respectively (Figure 5A). Among the 38 transformants obtained by PEG-mediated protoplast transformation, 35 showed a white phenotype and three remained orange. However, only seven white colonies were
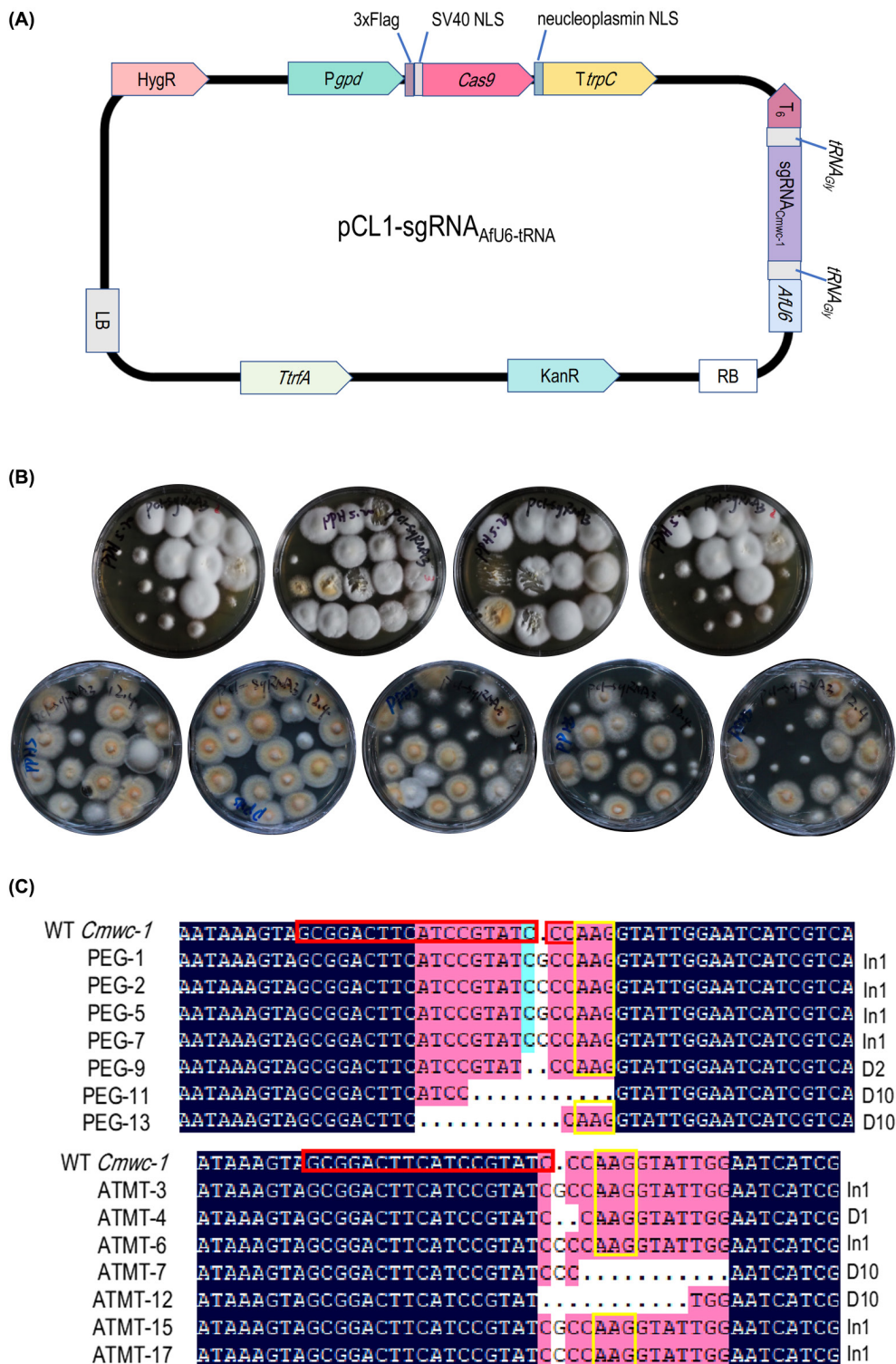
FIGURE 5 Comparison of efficiency between PEG-mediated protoplast transformation and ATMT. (A) Schematic of the plasmid, pLC1-sgRNA$_{Cmwc-1}$. (B) The colony of transformants obtained by PEG-mediated protoplast transformation (up) and ATMT (down). Both transformants were cultured on selective medium after light exposure. Bar = 3 cm. (C) DNA sequences of white colonies by PEG-mediated protoplast transformants and ATMT at *Cmwc-1* target sites. PEG-1, PEG-2, PEG-5, PEG-7, PEG-9, PEG-11, PEG-13, ATMT-3, ATMT-4, ATMT-6, ATMT-7, ATMT-12, ATMT-15 and ATMT-17 represent white colonies, and WT indicates the wild-type strain. In1, 1 bp insertion; D1, D2 and D10, 1 bp, 2 bp and 10 bp deletions. The yellow and red boxes indicate the PAM and guide sequences, respectively.

observed among the 70 transformants obtained by ATMT. DNA sequencing confirmed that the *Cmwc-1* genes of all white colonies were edited. PEG-mediated protoplast transformation was markedly more efficient than ATMT transformation in this system (Figure 5B, C).

DISCUSSION

Cordyceps militaris is one of the most economically valuable mushrooms worldwide. In this study, the AMA1-based CRISPR/Cas9 system was successfully used for gene editing in *C. militaris*. Plasmid loss under nonselective culture conditions enables recycling of the selectable marker and the next round of genetic engineering. Herein, editing efficiency was improved via a comparison of RNA Pol III promoters. Deletion of the target gene was achieved using a single sgRNA and HDR templates (Figure 6). PEG-mediated protoplast transformation had markedly higher efficiency than ATMT in *C. militaris*. To the best of our knowledge, this is the first description of an AMA1-based CRISPR/Cas9 system and precisely targeted gene deletion in mushrooms.

Based on the low probability of being inserted into the fungal genome during transformation and recycling of the selectable marker, several CRISPR/Cas9 systems based on AMA1 plasmids have been constructed in some filamentous fungi, such as *Talaromyces atroroseus* (Nielsen et al., 2017), *Penicillium chrysogenum* (Pohl et al., 2016) and *Aspergillus oryzae* (Katayama et al., 2019). In this study, AMA1-based CRISPR/Cas9 gene editing and plasmid loss by subculture under non-selective culture conditions were successfully performed for the first time in the ascomycetous mushroom, *C. militaris*. Theoretically, the mandatory recycling of genome-editing plasmids would facilitate the unlimited repeatability of genetic engineering steps.

This gene editing system is more useful for edible and medicinal fungi. Recycling the selectable marker and enabling the next round of genetic engineering will be helpful for genetic research as very few selectable markers are available in edible and medicinal fungi. Although the United States Department of Agriculture announced that the CRISPR/Cas9 gene-edited *Agaricus bisporus* fell outside of the genetically modified organism (GMO) legislation, some researchers reported that the edited strains may contain up to hundreds of base pairs of foreign DNAs in its genome (Kim & Kim, 2016). If foreign DNA is present, regulatory approval is required under current GMO legislation. AMA1 plasmids, which are characterized by extrachromosomal replication, have a low probability of being inserted into the fungal genome during transformation, which is useful for the generation of new cultivars.

Mutagenesis in the transformants mainly involved the deletion/insertion of 1–2 bases near PAM sites
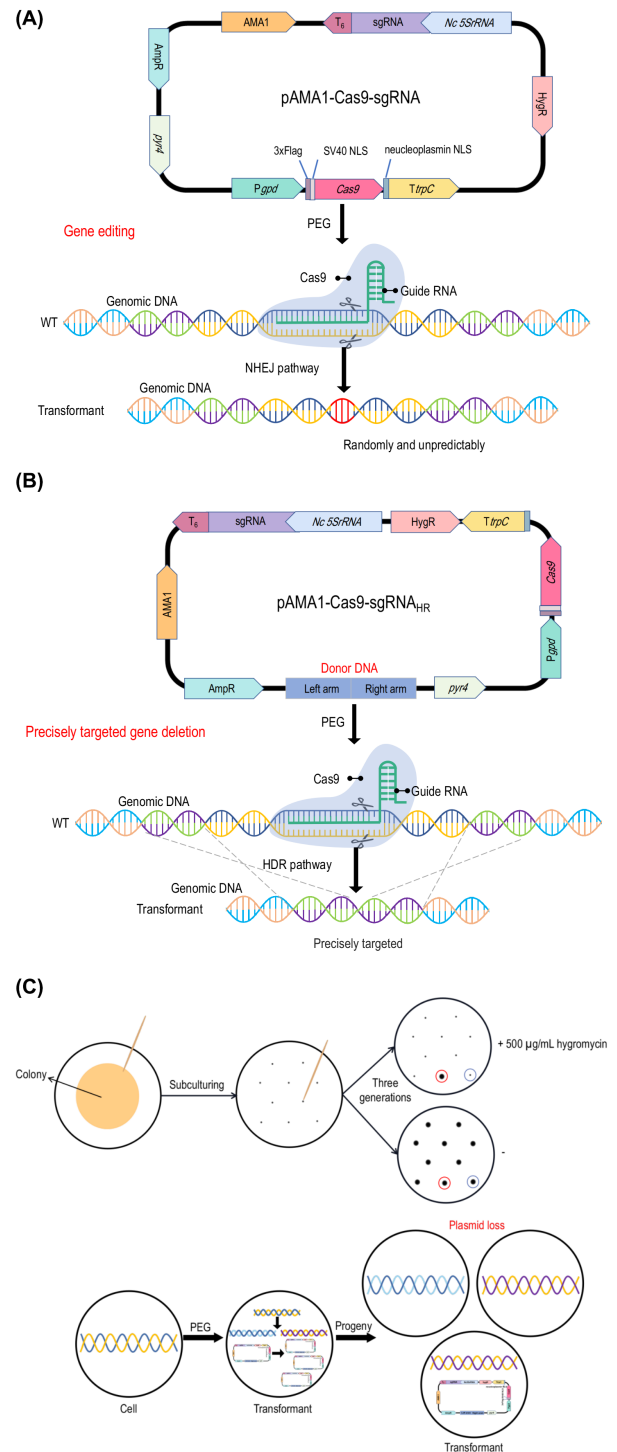
FIGURE 6 Efficient gene editing and targeted gene deletion by the AMA1-based CRISPR/Cas9 system, and plasmid loss under non-selective culture conditions. (A) Efficient gene editing based on the AMA1-based CRISPR/Cas9 system. (B) Precisely targeted gene deletion by the AMA1-based CRISPR/Cas9 system. (C) Plasmid loss under non-selective culture conditions. Red circles indicate the transformant containing *hyg* obtained by ATMT; blue circles indicate wild-type strains (control).

(Figure 1 and Figure S1); these mutations were random and unpredictable. Precisely targeted gene deletion is particularly important and has critical uses in

both strain breeding and functional genomics. In *G. lucidum*, the dual sgRNA-directed CRISPR/Cas9 system successfully achieved targeted gene deletion (Liu et al., 2020). Although Cas9 can create large deletions via NHEJ under paired sgRNAs, the implemented editing is still imprecise (Li & Huang, 2021). We also attempted targeted gene deletion in *C. militaris* using the dual sgRNA-directed CRISPR/Cas9 system; however, the efficiency was only 15% (data not shown). In this study, precisely targeted gene deletion was performed at an efficiency of 72% using an AMA1-based CRISPR/Cas9 system supplying donor DNAs. Precise indels can be created when DNA templates are provided to repair DNA DSBs via HDR. This technique could contribute to future research on gene function.

Using this AMA1-based CRISPR/Cas9 system, we achieved double-gene disruption in one step in *C. militaris*. *Cmwc-1* and *Cmvvd* were edited simultaneously with an efficiency of 10%. In *Acremonium chrysogenum*, double loci were simultaneously targeted only when supplied with HDR templates, and targeting multiple loci was inferred to be too lethal for the survival of *A. chrysogenum* C10 without repair templates as DSBs limit the survival of many fungi (Chen et al., 2020). Adding HDR templates could be attempted to improve the efficiency of multigene editing.

sgRNA expression is usually driven by RNA Pol III promoters to avoid post-transcriptional modifications (Cong et al., 2013). *N. crassa 5SrRNA* (E00012), downloaded from the *5SrRNA* database (http://combio.pl/rrna/), was selected as the promoter. The editing efficiencies for targeting *Cmwc-1* and *Cmvvd* were 55.1% and 88.89%, indicating the feasibility of using RNA Pol III promoters of heterologous *N. crassa 5SrRNA*. However, the double-gene-editing efficiency of this system was only 10%; thus, the RNA Pol III promoters were further optimized. Endogenous tRNAs from *C. militaris*, $tRNA_{Pro}$ and $tRNA_{Glu}$, and heterologous chimeric RNA Pol III promoters, *AfU6-tRNA_{Gly}*, from *A. fumigatus* Af293.1 (Chen et al., 2020) were compared. Endogenous $tRNA_{Pro}$ of *C. militaris* and heterologous chimeric RNA Pol III promoters *AfU6-tRNA_{Gly}* were found to be suitable as the editing efficiency was more than 80%. tRNA is a small non-coding RNA that is transcribed by RNA Pol III (Phizicky & Hopper, 2010). sgRNA transcription can be enhanced using tRNA (Xie et al., 2015), and the splicing mechanism of tRNA maturation ensures the precise release of sgRNA (Phizicky & Hopper, 2010). However, the types of tRNAs that can be effective as promoters should be verified experimentally. The *U6/tRNA* chimeric promoter was optimized in *A. chrysogenum* (Chen et al., 2020), and its efficiency was found to be similar to that of endogenous RNA Pol III promoters in *C. militaris*. It has been suggested that strict species specificity does not exist for RNA Pol III promoters.

Gene disruption, overexpression, and complementation have been performed using homologous recombination via ATMT in this fungus in our laboratory (Li et al., 2021; Xia et al., 2017; Yang et al., 2016; Zhang et al., 2020; Zheng et al., 2011). Initially, we attempted to perform CRISPR/Cas9 gene editing with pLC1-sgRNA$_{Cmwc-1}$ by ATMT; however, several failed attempts were documented (data not shown). Later, gene editing in *C. militaris* was achieved using PEG-mediated protoplast transformation. Thereafter, we compared the efficiency of PEG-mediated protoplast transformation and ATMT using plasmids harbouring the same Cas9 and sgRNA cassettes. PEG-mediated protoplast transformation was markedly more efficient than ATMT in *C. militaris* (Figure 5). Recently, Xu et al. (2021) found that the PEG-mediated protoplast transformation method using the CRISPR/Cas9 system remarkably shortened the time relative to ATMT in the endophytic fungus, *Pestalotiopsis fici*. This result seems reasonable as circular gene-targeting substrates are reported to be markedly more efficient for gene targeting than corresponding linear substrates in the transformation process (Nødvig et al., 2015).

In conclusion, our study provides an effective AMA1-based CRISPR/Cas9 system for highly efficient marker-free gene editing. RNA Pol III promoter optimization increases the editing efficiency in *C. militaris*. Moreover, the developed multiplex genome-editing systems and precisely targeted gene deletion can facilitate the application of the CRISPR/Cas9 technology in future studies of functional genes and strain breeding in the edible fungus, *C. militaris*.

AUTHOR CONTRIBUTIONS

D.C. conceived the idea, designed the study and revised the manuscript. M.G., W.X., L.M., W.F. and L.Q. performed the experiments. M.G. and W.X. wrote the manuscript and contributed equally to this work. All the authors have reviewed and approved the manuscript.

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CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The data supporting our findings are available in the manuscript file or from the corresponding author upon request.

The plasmids of pAMA1-Cas9-sgRNA$_{Cmwc-1}$ and pLC1-sgRNA$_{Cmwc-1}$ used in this study have been deposited at Addgene with the number of 81,087.

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REFERENCES

Barrangou, R. & Doudna, J. (2016) Applications of CRISPR technologies in research and beyond. *Nature Biotechnology*, 34, 933–941.

Boontawon, T., Nakazawa, T., Inoue, C., Osakabe, K. & Honda, Y. (2021) Efficient genome editing with CRISPR/Cas9 in *Pleurotus ostreatus*. *AMB Express*, 11, 30.

Chen, B.X., Wei, T., Wei, Y.Z., Yun, F., Kang, L.Z., Tang, H.B. et al. (2018) Efficient CRISPR-Cas9 gene disruption system in edible-medicinal mushroom *Cordyceps militaris*. *Frontiers in Microbiology*, 9, 1157.

Chen, C., Liu, J.J., Duan, C.B., Pan, Y.Y., and Liu, G. (2020) Improvement of the CRISPR-Cas9 mediated gene disruption and large DNA fragment deletion based on a chimeric promoter in *Acremonium chrysogenum*. *Fungal Genetics and Biology,* 134: 103279.

Choi, J., Chen, W., Suiter, C.C., Lee, C., Chardon, F.M., Yang, W. et al. (2021) Precise genomic deletions using paired prime editing. *Nature Biotechnology*, 40, 218–226. https://doi.org/10.1038/s41587-021-01025-z

Cong, L., Ran, F.A., Cox, D., Lin, S.L., Barretto, R., Habib, N. et al. (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science*, 339, 819–823.

Cunningham, K.G., Manson, W., Spring, F.S. & Hutchinson, S.A. (1950) Cordycepin, a metabolic product isolated from cultures of *Cordyceps militaris* (Linn.) link. *Nature*, 166, 949.

Fu, M.J. (2005) Study on the carotenoid produced from *Cordyceps militaris* L. *Journal of Food Science and Biotechnology*, 24, 107–110.

Gai, X.J. & Zhang, Y.S. (1992) Study of extracellular polysaccharide from *Cordyceps militaris* (L.) link I. purification and structural study of galactomannan (CM-I). *Mycosystema*, 4, 300–307.

Gems, D., Johnstone, I.L. & Clutterbuck, A.J. (1991) An autonomously replicating plasmid transforms *Aspergillus nidulans* at high frequency. *Gene*, 98, 61–67.

Hsu, P.D., Lander, E.S. & Zhang, F. (2014) Development and applications of CRISPR-Cas9 for genome engineering. *Cell*, 157, 1262–1278.

Jan Vonk, P., Escobar, N., Wosten, H.A.B., Lugones, L.G. & Ohm, R.A. (2019) High-throughput targeted gene deletion in the model mushroom *Schizophyllum commune* using pre-assembled Cas9 ribonucleoproteins. *Scientific Reports*, 9, 7632.

Katayama, T., Nakamura, H., Zhang, Y., Pascal, A., Fujii, W. & Maruyama, J.I. (2019) Forced recycling of an AMA1-based genome-editing plasmid allows for efficient multiple gene deletion/integration in the industrial filamentous fungus *Aspergillus oryzae*. *Applied and Environmental Microbiology*, 85, e01896–e01818.

Kim, J. & Kim, J.S. (2016) Bypassing GMO regulations with CRISPR gene editing. *Nature Biotechnology*, 34, 1014–1015.

Li, X., Wang, F., Liu, M.Q. & Dong, C.H. (2021) Hydrophobin CmHYD1 is involved in conidiation, infection and primordium formation, and regulated by GATA transcription factor CmAreA in edible fungus, *Cordyceps militaris*. *Journal of Fungi*, 7, 674.

Li, X.Y. & Huang, X.X. (2021) Towards precise large genomic fragment deletion. *Trends in Genetics*, 38, 214–215. https://doi.org/10.1016/j.tig.2021.11.004

Liu, K., Sun, B., You, H., Tu, J.L., Yu, X.Y., Zhao, P. et al. (2020) Dual sgRNA-directed gene deletion in basidiomycete *Ganoderma lucidum* using the CRISPR/Cas9 system. *Microbial Biotechnology*, 13, 386–396.

Lou, H., Lin, J., Guo, L., Wang, X. & Zhao, R. (2019) Advances in research on *Cordyceps militaris* degeneration. *Applied Microbiology and Biotechnology*, 103, 7835–7841.

Lou, H.W., Ye, Z.W., Yun, F., Lin, J.F., Guo, L.Q., Chen, B.X. et al. (2018) Targeted gene deletion in *Cordyceps militaris* using the split-marker approach. *Molecular Biotechnology*, 60, 380–385.

Mefferd, A.L., Kornepati, A.V., Bogerd, H.P., Kennedy, E.M. & Cullen, B.R. (2015) Expression of CRISPR/Cas single guide RNAs using small tRNA promoters. *RNA*, 21, 1683–1689.

Nallathamby, N., Guan-Serm, L., Vidyadaran, S., Abd Malek, N., Ramam, J. & Sabaratnam, V. (2015) Ergosterol of *Cordyceps militaris* attenuates lps induced inflammation in bv2 microglia cells. *Natural Product Communications*, 10, 885–886.

Nielsen, M.L., Isbrandt, T., Rasmussen, K.B., Thrane, U., Hoof, J.B., Larsen, T.O. & Mortensen, U.H. (2017) Genes linked to production of secondary metabolites in *Talaromyces atroroseus* revealed using CRISPR-Cas9. *PLoS One*, 12, e0169712.

Nødvig, C.S., Nielsen, J.B., Kogle, M.E. & Mortensen, U.H. (2015) A CRISPR-Cas9 system for genetic engineering of filamentous fungi. *PLoS One*, 10, e0133085.

Phizicky, E.M. & Hopper, A.K. (2010) tRNA biology charges to the front. *Genes and Development*, 24, 1832–1860.

Pohl, C., Kiel, J.A., Driessen, A.J., Bovenberg, R.A.L. & Nygård, Y. (2016) CRISPR/Cas9 based genome editing of *Penicillium chrysogenum*. *ACS Synthetic Biology*, 5, 754–764.

Qin, H., Xiao, H., Zou, G., Zhou, Z.H. & Zhong, J.J. (2017) CRISPR-Cas9 assisted gene disruption in the higher fungus *Ganoderma* species. *Process Biochemistry*, 56, 57–61.

Sugano, S.S., Suzuki, H., Shimokita, E., Chiba, H., Noji, S., Osakabe, Y. et al. (2017) Genome editing in the mushroom-forming basidiomycete *Coprinopsis cinerea*, optimized by a high-throughput transformation system. *Scientific Reports*, 7, 1260.

Sun, S.J., Deng, C.H., Zhang, L.Y. & Hu, K.H. (2017) Molecular analysis and biochemical characteristics of degenerated strains of *Cordyceps militaris*. *Archives of Microbiology*, 199, 939–944.

Waltz, E. (2016) Gene-edited CRISPR mushroom escapes US regulation. *Nature*, 532, 293.

Wang, P.A., Xiao, H. & Zhong, J.J. (2020) CRISPR-Cas9 assisted functional gene editing in the mushroom *Ganoderma lucidum*. *Applied Microbiology and Biotechnology*, 104, 1661–1671.

Wang, Q. & Coleman, J.J. (2019) Progress and challenges: development and implementation of CRISPR/ Cas9 technology in filamentous fungi. *Computational and Structural Biotechnology Journal*, 17, 761–769.

Wang, T., Yue, S., Jin, Y., Wei, H. & Lu, L. (2021) Advances allowing feasible *pyrG* gene editing by a CRISPR-Cas9 system for the edible mushroom *Pleurotus eryngii*. *Fungal Genetics and Biology*, 147, 103509.

Weyda, I., Yang, L., Vang, J., Ahring, B.K., Lübeck, M. & Lübeck, P.S. (2017) A comparison of *Agrobacterium*-mediated transformation and protoplast-mediated transformation with CRISPR-Cas9 and bipartite gene targeting substrates, as effective gene targeting tools for *Aspergillus carbonarius*. *Journal of Microbiological Methods*, 135, 26–34.

Wohlers, T.E. (2015) The role of risk perception and political culture: a comparative study of regulating genetically modified food. In: Mercantini, J.M. & Faucher, C. (Eds.) *Risk and cognition. Intelligent systems reference library*, Vol. 80. Berlin, Heidelberg: Springer.

Xia, Y.L., Luo, F.F., Shang, Y.F., Chen, P.L., Lu, Y.Z. & Wang, C.S. (2017) Fungal cordycepin biosynthesis is coupled with the production of the safeguard molecule pentostatin. *Cell Chemical Biology*, 24, 1479–1489.

Xie, K., Minkenberg, B. & Yang, Y. (2015) Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. *Proceedings of the National Academy of Sciences of the United States of America*, 112, 3570–3575.

Xu, X.R., Huang, R.Y. & Yin, W.B. (2021) An optimized and efficient CRISPR/Cas9 system for the endophytic fungus *Pestalotiopsis fici*. *Journal of Fungi*, 7, 809.

Yang, T., Guo, M.M., Yang, H.J., Guo, S.P. & Dong, C.H. (2016) The blue-light receptor CmWC-1 mediates fruit body development and secondary metabolism in *Cordyceps militaris*. *Applied Microbiology and Biotechnology*, 100, 743–755.

Zhang, C., Meng, X., Wei, X. & Lu, L. (2016) Highly efficient CRISPR mutagenesis by microhomology-mediated end joining in *Aspergillus fumigatus*. *Fungal Genetics and Biology*, 86, 47–57.

Zhang, J.J., Wang, F., Liu, K.B., Liu, Q., Yang, Y. & Dong, C.H. (2018) Heat and light stresses affect metabolite production in the fruit body of the medicinal mushroom *Cordyceps militaris*. *Applied Microbiology and Biotechnology*, 102, 4523–4533.

Zhang, J.J., Wang, F., Yang, Y., Wang, Y. & Dong, C.H. (2020) CmVVD is involved in fruiting body development and carotenoid production and the transcriptional linkage among three blue-light receptors in edible fungus *Cordyceps militaris*. *Environmental Microbiology*, 22, 466–482.

Zheng, Z., Huang, C.H., Cao, L., Xie, C.H. & Han, R.C. (2011) *Agrobacterium tumefaciens*-mediated transformation as a tool for insertional mutagenesis in medicinal fungus *Cordyceps militaris*. *Fungal Biology*, 115, 265–274.

Zou, G., Xiao, M.L., Chai, S.X., Zhu, Z.H., Wang, Y. & Zhou, Z.H. (2021) Efficient genome editing in filamentous fungi *via* an improved CRISPR-Cas9 ribonucleoprotein method facilitated by chemical reagents. *Microbial Biotechnology*, 14, 2343–2355.

SUPPORTING INFORMATION

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