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CRISPR-Cas9 induces point mutation in the mucormycosis fungus *Rhizopus delemar*

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Abstract

Rhizopus delemar causes devastating mucormycosis in immunodeficient individuals. Despite its medical importance, *R. delemar* remains understudied largely due to the lack of available genetic markers, the presence of multiple gene copies due to genome duplication, and mitotically unstable transformants resulting from conventional and limited genetic approaches. The clustered regularly interspaced short palindromic repeat (CRISPR)-associated nuclease 9 (Cas9) system induces efficient homologous and non-homologous break points and generates individual and multiple mutant alleles without requiring selective marker genes in a wide variety of organisms including fungi. Here, we have successfully adapted this technology for inducing gene-specific single nucleotide (nt) deletions in two clinical strains of *R. delemar*: FGSC-9543 and CDC-8219. For comparative reasons, we first screened for spontaneous uracil auxotrophic mutants resistant to 5-fluoroorotic acid (5-FOA) and obtained one substitution (f1) mutation in the FGSC-9543 strain and one deletion (f2) mutation in the CDC-8219 strain. The f2 mutant was then successfully complemented with a *pyrF-dpl200* marker gene. We then introduced a vector pmCas9:trRNA-gRNA that expresses both Cas9 endonuclease and *pyrF*-specific gRNA into FGSC-9543 and CDC-8219 strains and obtained 34 and 42 5-FOA resistant isolates, respectively. Candidate transformants were successively transferred eight times by propagating hyphal tips prior to genotype characterization. Sequencing of the amplified *pyrF* allele in all transformants tested revealed a single nucleotide (nt) deletion at the 4th nucleotide before the protospacer adjacent motif (PAM) sequence, which is consistent with CRISPR-Cas9 induced gene mutation through non-homologous end joining (NHEJ). Our study provides a new research tool for investigating molecular pathogenesis mechanisms of *R. delemar* while also highlighting the utilization of CRISPR-Cas9 technology for generating specific mutants of Mucorales fungi.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fgb.2018.12.002>.

Keywords

5-FOA resistant mutation; *PyrF* and *PyrG*; Gene-specific mutagenesis; Mucormycosis pathogen

1. Introduction

Mucormycosis is a highly invasive and life-threatening fungal infection that occurs in patients with an underlying immune deficiency due to hematologic diseases, diabetic ketoacidosis, or immunosuppressive medication following organ transplantation. The infection is caused by Mucorales fungi, including the genera of *Rhizopus*, *Mucor*, and *Rhizomucor* (Hibbett et al., 2007). *Rhizopus delemar* (also known as *R. oryzae*) is the predominant etiologic agent of mucormycosis, responsible for ~70% of clinical cases (Gomes et al., 2011; Spellberg et al., 2005). Because of the increasing prevalence of immune-compromised individuals, the number of patients at risk and those succumbing to fatal infection have dramatically increased. From 2007 to 2009, six fatal mucormycosis deaths were reported in a pediatric hospital in New Orleans. The deceased patients ranged in age from premature infants to a 13-year-old child (Duffy et al., 2014). Fatal mucormycosis also occurred recently among organ transplant recipients with multiple fatalities in two university hospitals in Pittsburgh (Novosad et al., 2016; Valle, 2017) and in the Queen Mary Hospital in Hong Kong (Cheng et al., 2016). Although individual cases tend to occur more frequently, these outbreaks underscored the significance of the infection.

Despite the increased incidence and severity of the disease, very limited diagnostic and treatment options are available for mucormycosis. Although genetic approaches such as transformation-mediated gene disruption, gene suppression, and other gene and genome manipulations are common for studying molecular pathogenesis, genetic tools to study Mucorales fungi, especially *R. delemar*, are limited. *R. delemar* has aseptate or coenocytic hyphae and produces multinucleated spore propagules. A previous study has also suggested that an ancient genome duplication event resulted in many genes in multiple copies in this fungus (Ma et al., 2009). Together, these characteristics presented many challenges, such as inefficiency and difficulty recovering null mutants, which ultimately complicates studies of *R. delemar* virulence mechanisms. Therefore, there is an urgent need to develop new methodology necessary for the study and management of the infection.

Various approaches aimed at efficient introduction of precise, targeted genome alterations in living cells are now available in many organisms, including mammals, plants, and fungi. These approaches employ meganucleases, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly inter-spaced short palindromic repeat (CRISPR)-CRISPR-associated nuclease 9 (Cas9). Among those, CRISPR-Cas9 is the most innovative, since it uses a simple base-pairing rule between an engineered RNA and the target DNA site [reviewed in (Harrison et al., 2014)]. The CRISPR-Cas9 system utilizes the RNA-guided Cas9 nuclease activity to introduce sequence-specific double strand breaks (DSBs) and targeted genome editing [reviewed in (Sander and Joung, 2014)]. The system involves two components: a Cas9 nuclease and a guide RNA (gRNA) consisting of a fusion between a CRISPR RNA (crRNA) and a fixed trans-activating crRNA

(tracrRNA). Twenty nucleotides (nts) at the 5' end of gRNA that correspond to the protospacer portion of the crRNA immediately adjacent to the NGG protospacer adjacent-motif (PAM) site) direct Cas9 to a specific DNA target site [reviewed in (Sander and Joung, 2014)]. Targeted genome editing using CRISPR-Cas9 provides a general method for inducing targeted deletions, insertions, and precise sequence changes in at least 22 organisms and cell types, including many fungal species (Mitchell, 2017).

The ability of CRISPR-Cas9 technology to efficiently introduce specific and precise DNA sequence editing and its successful adaptation in a wide array of fungal species offers promise as an advanced tool for adapting to *R. delemar* research. The ability to induce homologous and nonhomologous break points will facilitate the selection of mitotically stable transformants and the generation of individual or multiple mutant alleles without requiring selection marker genes thereby enabling the study of multi-copy gene functions. Encouragingly, a recent report showed the successful application of CRISPR-Cas9 technology in *Mucor circinelloides*, a related but different mucormycosis pathogen (Nagy et al., 2017). Here, we report the successful adaptation of the CRISPR-Cas9 system to generate uracil auxotrophic mutants in *R. delemar*. By utilizing the plasmid pmCas9:tRNAp-gRNA that expresses both Cas9 endonuclease and gRNA, we generated mutant strains specific to the *pyrF* gene. This technology holds great potential for the generation of additional auxotrophic mutants and further development of auxotrophic markers for genetic manipulation in *R. delemar*. Such developments will greatly improve the genetic tractability and facilitate the disruption of individual or even multiple genes in this fungus.

2. Results

2.1. Isolation and complementation of 5-FOA resistant spontaneous mutants

To adapt CRISPR-Cas9-mediated gene editing for *R. delemar*, we began our studies by identifying auxotrophic mutant strains that could be used as the recipient strains. We found that *R. delemar* exhibits greater resistance to hygromycin (200 µg/ml), nourseothricin (300 µg/ml), and G418 (300 µg/ml) that adversely affects the prospects of using these drugs as selection markers, although hygromycin resistance was previously utilized in the genetic transformation (Zhang et al., 2015). Auxotrophic mutant strains of *R. delemar* were rarely identified, but one uracil auxotrophic mutant, M16, was previously reported (Skory and Ibrahim, 2007). Since ura⁻ strains can be selected for resistance to 5-fluoroorotic acid (5-FOA), we carried out our screen using two clinical strains of *R. delemar*. FGSC-9543, also known as RA 99–880, is a reference strain used in the genome-sequencing project by the Broad Institute of MIT, and CDC-8219 was isolated from a local pediatric patient succumbed to fatal *R. delemar* infection in 2009 (Duffy et al., 2014). In the initial screening, six 5-FOA (1 g/l) resistant colonies out of each strain were obtained (Fig. 1A). Following the repeated colony transfer (8x), four remaining isolates from each strain (8 total) were subjected to further analysis.

Although *R. delemar* has undergone genome duplication resulting in many genes in multiple copies (Ma et al., 2009), genes such as *pyrF* encoding orotate phosphoribosyl transferase and *pyrG* encoding orotidine-5'-monophosphate decarboxylase are each present as a single copy. Mutations in the *pyrF* gene and/or the *pyrG* gene disrupt the uridine biosynthetic

pathway, resulting in resistance to 5-FOA (Boeke et al., 1987; Skory and Ibrahim, 2007). To examine if these isolates had any mutations in *pyrF* and *pyrG* genes, they were PCR amplified with primers PW1819/PW1828 (~3.0-kb) and PW1829/PW1833 (~1.8-kb) respectively. Sequencing of the 3.0-kb fragment containing the 5' UTR, the 654 nt *pyrF* open reading frame (ORF), and 3' UTR revealed that one of the FGSC-9543 isolates, named f1, had a single C to T transition (255C > T), resulting in L85 to F85 exchange (L85F) (Fig. 1B). And, one of the four CDC-8219 isolates, f2, had a deletion of TC (91–92 TC), disrupting the *pyrF* ORF (Fig. 1B). The 1.8-kb *pyrG* ORF (with 5' but without 3' UTR regions) was intact in these two strains, as well as in the other 5-FOA resistant strains with the intact *pyrF* ORF, suggesting that additional mechanisms may also be involved in 5-FOA resistance. Sequencing results also indicated that the *pyrF* and *pyrG* genes are highly conserved between FGSC-9543 and CDC-8219 strains, sharing ~99.5% identity in nt sequences and 100% identity in amino acid sequences.

To further test whether 5-FOA resistance was due to the mutation in the *pyrF* gene, the CDC-8219f2 isolate was complemented with a *pyrF-dpl200* marker gene containing a 200-bp sequence of the 5'-UTR sequence inserted at the 3' UTR region of the wild type (WT) *pyrF* gene (Sup. Fig. S1). Transformation was performed using the biolistic transformation approach. The resulting transformants TF1 and TF3 showed expected hyphae growth on PDA, MMC -uracil, and MMC + 5-FOA medium in comparison to the control strains (Fig. 1C) before passaged on MMC ura- medium six times and grown for genomic DNA extraction. Sequencing of the 217-bp PCR fragment amplified with primers PW1972 and PW1945 that spans the *pyrF*(91–92 TC) region (Sup. Fig. S2) revealed the presence of mixed DNA products in transformants TF1 and TF3 (Fig. 1D, top chromatograms). This result was verified by sequencing individual cloned PCR products that contain either the partial WT *pyrF* sequence from *pyrF-dpl200* or the *pyrF*(91–92 TC) allele (Fig. 1D, bottom chromatograms). Further PCR analysis with primers PW2015 and PW2050 yielded one 380- or/and one 610-bp fragments, corresponding to the endogenous *pyrF* gene or/and the *pyrF-dpl200* sequence, respectively (Fig. 1E). Collectively, the results indicated that 5-FOA resistance was due to the mutation in the *pyrF* gene and its complementation occurred as an ectopic integration event.

2.2. Consideration of CRISPR-Cas9 plasmid vectors

The CRISPR-Cas9 system uses a type II RNA-guided endonuclease, Cas9, from *Streptococcus pyogenes* and a custom designed genome targeting CRISPR gRNA, consisting of a crRNA and a fixed tracrRNA. The 20-nts at the 5' end of the gRNA that corresponds to the protospacer portion of the crRNA direct the Cas9 endonuclease to specific DNA target sites. In most CRISPR-Cas9 applications, the *CAS9* gene, fused with a nuclear localization signal from Simian virus 40 (SV40nls), is a codon-optimized version that was originally constructed for expression in mammalian cells (Mali et al., 2013). A version fused with an endogenous promoter, either *GAL1* or *TEF1*, and an SV40nls, was used in the CRISPR-Cas system for the budding yeast *Saccharomyces cerevisiae*. In that study, the constitutive expression of Cas9 (and gRNA) yielded a near 100% donor DNA recombination frequency (DiCarlo et al., 2013). In contrast to Cas9 expression that requires an RNA polymerase II (Pol II) promoter, gRNA expression requires a Pol III promoter to

minimize any post-transcriptional modifications. In addition to the promoter, gRNA expression requires a gRNA structural component and the TTTTTT terminator sequence. In systems tested so far, eukaryotic U6 and U3 snRNA promoters were often used to drive gRNA transcription. Since no such information is available for *R. delemar*, we sought to test existing CRISPR-Cas9 constructs developed for gene editing of other filamentous fungi, such as pPDC-Cas9 (Liu et al., 2015) and pmCas9:tRNA-gRNA (Liang et al., 2018; Wang, 2018).

pPDC-Cas9 contains a *Ppdc*-toCas9-*Tpdc* cassette optimized for *Trichoderma reesei* and does not contain a gRNA expression component (Liu et al., 2015). pmCas9:tRNA-gRNA, on the other hand, contains a codon-optimized Cas9 expression cassette driven by a constitutively active translation elongation factor promoter (Arazoe et al., 2014; Arazoe et al., 2013) and a glutamyl (Gln)-tRNA-gRNA expression cassette (Liang et al., 2018) (Fig. 2). The Gln-tRNA sequence exhibiting a Pol III promoter activity was identified from the rice false smut fungus *Ustilagoidea virens* (oryzae) genome using the tRNAscan-SE program (Liang et al., 2018). The combination of two expression elements in one plasmid and the simple insertion of gRNA sequences proved to be convenient in previous reports of CRISPR-Cas9-mediated gene editing in several fungi (Liang et al., 2018; Wang, 2018). We thus decided to test this plasmid for CRISPR-Cas9 in *R. delemar*.

2.3. CRISPR-Cas induces 5-FOA resistant mutations

To design gRNA primers targeting the *pyrF* gene, we used flyCRISPR (<http://flycrispr.molbio.wisc.edu>). Complementary gRNA primers specific to the 5' and 3' regions of the 654 nts *pyrF* gene were synthesized (Table 1), annealed and used to construct pmCas9:tRNA-*pyrF1* and pmCas9:tRNA-*pyrF2* plasmids, respectively. Primers PW1947/PW1948 for gRNA #1 (*pyrF1*) were specific to the nts 40–59 of the *pyrF* gene, while primers PW2014/PW2015 for gRNA #2 (*pyrF2*) targeted at the nts 484–503 (Sup. Fig. S2). Both targets are located within the exon regions of the gene. To attempt CRISPR-Cas9-mediated *pyrF* gene replacement, a 2.5-kb *pyrF::HYG* fragment obtained by PCR amplification with primers PW1823 and PW1827 was also included as donor template DNA (Sup. Fig. S2). In a typical transformation, 0.5 µg each plasmid DNA and 0.2 µg homologous donor DNA was introduced into 50 µl [1×10^6 colony forming unit (cfu)/ml] swollen spores by electroporation. Plasmid DNA was first linearized by KpnI restriction and cleaned with a DNA cleanup kit (Qiagen). Donor DNA was amplified by PCR and cleaned using QIAquick (Qiagen). Following an incubation time of 5–10 days at 30°C, approximately 5–8 colonies could be seen in one transformation plate containing 5-FOA (2 g/l) and hygromycin (200 µg/ml) that received plasmids and donor DNA, but none or few colonies from the plates that received only donor DNA, which were not followed by characterization (Fig. 3). Based on ~5 initial putative transformants per 50 µl (1×10^6 cfu/ml) and the spontaneous mutation result of obtaining 12 putative transformants from a total of 20 plates (200 µl each plate, 1×10^8 cfu/ml), pmCas9:tRNA-gRNA/electroporation induced significantly more, at least 166-fold (2000:12), *pyrF* mutants than the spontaneous approach. Additionally, none or few transformants were found in electroporation with only the homologous donor DNA (Fig. 3) and these transformants often failed to grow during

repeated culture transfers. Collectively, these evidence suggested that the mutation could be mediated by CRISPR-Cas9.

2.4. Evidence for CRISPR-Cas9-mediated *pyrF* gene deletion

We then characterized the 5-FOA resistant isolates obtained above to examine if they were indeed mutants mediated by CRISPR-Cas9. *R. delemar* produces multi-nucleated spores and no method of inducing uninuclear spores is available. To circumvent this obstacle, we stained the coenocytic hyphae with 4', 6-diamidino-2-phenylindole (DAPI) and observed that the distances between two nuclei are longer than those in spores (Sup. Fig. S3). We thus decided to transfer the hyphal tips in order to enrich or purify nuclei with the edited *pyrF* gene allele. To do this, we cut and transferred hyphal tips of ~0.1-cubic mm with a sharp scalpel under a dissecting microscope. We carefully transferred hyphal tips growing deep into the agar medium and transferred multiple tips of each isolate to ensure successful re-culturing. After ten such transfers, one FGSC-9543 and four CDC-8219 transformants that retained 5-FOA resistance were grown in liquid YPD containing 5-FOA (1 g/l) for DNA extraction.

In CRISPR-Cas9-mediated gene editing, multiple gRNA constructs can be used in a single transformation to specifically edit the gene of the interest. Donor DNA can also be included to enable homology directed repair (HDR) gene replacement. We included pmCas9-tRNA-*pyrF1*, pmCas9-tRNA-*pyrF2*, and the *pyrF::HYG* fragment, intending to replace the WT *pyrF* gene with the *pyrF::HYG* allele (Sup. Fig. S2). To identify whether any mutations occurred, primers PW1972 and PW2050 and primers PW1860 and PW1861 and were used to amplify the *pyrFORF* and the partial *HYG* fragment, respectively (Table 1 and Sup. Fig. S2). Intriguingly, PCR amplification with primers PW1972/PW2050 generated a wild-type-like 0.9-kb fragment in all five transformants and primers PW1860/PW1861 failed to yield any fragments. Additional primers PW1862/PW1863 also specific to the *HYG* resistant gene cassette was used but failed to yield any DNA fragment (data not shown). These results suggested that 5-FOA resistant mutation was less likely to be the result of homology directed repair (HDR) but rather non-homologous end joining (NHEJ). To test this reasoning, both the 0.9-kb PCR product and the clone insert (pCR2.1, Invitrogen) were subject to sequencing analysis. Sequencing data revealed that there was only a single nt deletion in the *pyrF* allele and that the deletion occurred at a site 4-nt away from the PAM site of the sequence targeted by pmCas9:tRNA-*pyrF1* (Fig. 4, one FGSC-9543 and two CDC-9219 isolates shown). This pattern appearing in all five 5-FOA mutants (one for FGSC-9543 and four for CDC-8219) is consistent with a CRISPR-Cas9 induced NHEJ event.

To explore why NHEJ occurred at the region targeted only by pmCas9:tRNA-*pyrF1*, but not that by pmCas9:tRNA-*pyrF2*, and there was no HDR, we re-examined both gRNA target sequences. As gRNA was originally designed with the flyCRISPR program developed for *Drosophila* (flycrispr.molbio.wisc.edu), we neglected to further assess their hypothetical on-target efficiency that might account for our unexpected results. We estimated on-target efficiency using the CRISPR-Cas9 guide RNA design checker (www.idtdna.com) and found that pmCas9:tRNA-*pyrF1* has a moderate efficiency of 59% and pmCas9:tRNA-*pyrF2* has

an efficiency of 36%. Although this efficiency assessment was not *R. delemar*-specific, it might provide an explanation that only pmCas9:tRNA-*pyrF1*, but not pmCas9:tRNA-*pyrF2*, was able to target the *pyrF* sequence resulting in NHEJ. Taken together, these results suggested that pmCas9:tRNA-*pyrF1* induced point mutation of the *pyrF* gene in both FGSC-9543 and CDC-8219 strains.

3. Discussion

Members of the Mucorales including *Rhizopus* and *Mucor* species cause fulminant, life-threatening infections in patients with immune deficiencies. Because of the increasing prevalence of diabetes mellitus, cancer, and organ transplantation, the number of patients at risk for mucormycosis is dramatically increasing. Despite surgical debridement and adjunctive antifungal therapy, the overall mortality rate remains formidably higher than 50% and approaches 100% in individuals with disseminated diseases or persistent neutropenia (Spellberg et al., 2005). *Rhizopus* species are highly heterogeneous with aseptate hyphae and multi-nucleated spores. The limited preliminary genetic and genomic studies have provided an early glimpse into the genetic makeup of *R. delemar*. In a comparative study of selected plant and human pathogenic fungi, *R. delemar* was found to contain multiple copies for most of its genes. Among 62 genes identified, 32 have at least one or more copies (32/62), in comparison to 19/83 for *S. cerevisiae*, 4/67 for *Candida albicans*, and 4/73 for *Aspergillus fumigatus* (Rispaill et al., 2009). A more recent study has revealed that *R. delemar* might have undergone whole genome duplication (WGD) (Ma et al., 2009). These studies provided an explanation for the presence of multiple gene copies in *R. delemar*, and they also indicated that this fungus is not as genetically tractable as other fungal pathogens.

At the present time, approaches such as transformation-mediated gene disruption, gene suppression or other gene and genome manipulation approaches are commonly employed for studying mechanisms of pathogenesis. However, few genes have been subjected to such studies in *R. delemar* because of the difficulty in generating stable transformants. PEG/CaCl₂-protoplast-mediated transformation, electroporation, or *Agrobacterium tumefaciens* T-DNA have all been attempted for transformation, but the efficiency and stability remain problematic (Ibrahim et al., 2010; Meussen et al., 2012; Xu et al., 2014). Recent studies have demonstrated that CRISPR-Cas9 is the most innovative technology that offers precisely targeted and highly efficient editing of genome sequences [reviewed in (Mitchell, 2017; Sander and Joung, 2014)] and its promise as an advanced tool to study the mechanism of pathogenesis in *R. delemar* prompted our research effort.

Due to the lack of research resources available for *R. delemar*, we initiated our studies by identifying *bona fide* promoter sequences for expressing Cas9 and gRNA. We profiled extracellular small RNA (ex-sRNA) in an attempt to identify a noncoding RNA promoter polymerase from *R. delemar* that has the Pol III RNA polymerase promoter activity (Liu et al., 2018). We also tested several DNA plasmids including pPDC-Cas9 and pCas9:tRp-gRNA for transformation of *R. delemar*. pPDC-Cas9 contains the *T. reesei* codon optimized Cas9 driven by the Gpd1 promoter and the TrpC terminator sequences of *Neurospora crassa* (Liu et al., 2015), but this vector does not contain any components for gRNA expression. pmCas9:tRNAp-gRNA was developed for several fungi including *U. virens* (Liang et al.,

2018) and *Magnaporthe oryzae* (Keili Zhong and Zhengguang Zhang, personal communication) and it contains both the *CAS9* gene expressing Cas9 and a 75-nt Gln tRNA exhibiting a Pol III RNA polymerase promoter activity. This vector has also been modified for CRISPR-Cas9-mediated gene editing in *C. neoformans* (Wang, 2018). We therefore focused on pmCas9:tRNA^p-gRNA and demonstrated that this vector could induce specific mutations by NHEJ in both clinical strains of *R. delemar*.

The successful adaptation of CRISPR-Cas9 technology for gene editing in *R. delemar* is significant as it holds great promise as an advanced tool for genetic studies into *R. delemar* virulence determinants. We demonstrated the ability of CRISPR-Cas9 to induce point mutation in this study and ongoing adaptation of CRISPR-Cas9 technology, using multiple gRNAs for NHEJ-mediated gene editing, in combination with knock-out alleles for homologous donor recombination, should enable us to generate multiple mutant alleles simultaneously, which would be extremely advantageous in *R. delemar* since there are multiple copies of some genes involved in virulence. However, issues regarding the fate of the introduced plasmid sequences that potentially affect the phenotypes of the transformants remain to be addressed. The approach of ribonucleotide protein complex-mediated (RNP) method for transient expression should mitigate this concern. Moreover, any off-target assessment will also be needed. Therefore, continued adaptation and refinement of CRISPR-Cas9 technology will provide us with an advanced research tool in the elucidation of pathogenic mechanisms in *R. delemar*.

4. Materials and methods

4.1. Fungal strains and DNA plasmids

R. delemar FGSC-9543 and CDC-8219 strains were maintained in potato dextrose agar (PDA) and grown in PDA+ medium (PDA plus 1% yeast extract, 1% peptone, and 1% glycerol) for spore production (Liu et al., 2018). MMC medium (1% casamino acids, 0.05% yeast nitrogen base without amino acids and ammonium sulfate, 2% glucose, pH 3.8) was used for the characterization of *pyrF*-mediated phenotypes. DNA plasmids pPDC-Cas9 and pCas9:tRNA^p-gRNA were the gifts from Drs. Gen Zou and Zhengguang Zhang, respectively. pmCas9:tRNA^p-gRNA (also known as pmCas9 (Liang et al., 2018) contains the *Cas9* gene optimized for fungal expression (Arazoe et al., 2014; Arazoe et al., 2013) and a gRNA expression cassette using the 75-nt Gln tRNA from the rice false smut fungus *U. virens* that exhibits Pol III RNA polymerase promoter activity (Liang et al., 2018). Primer sequences were listed in Table 1.

4.2. Generation of 5-FOA resistant strains and mutant complementation with a *pyrF*-*dpl200* marker gene

A direct selective approach was employed to identify 5-FOA/*ura*-auxotrophy. We found that potato dextrose agar fortified with 1% yeast extract, 1% peptone, and 1% glycerol enhances abundant sporulation for *R. delemar*. Spores were harvested from one-week old cultures of FGSC-9543 and CDC-8219 strains, washed with sterile distilled water three times, and spread (0.2 ml 1×10^8 cfu/ml) onto the synthetic minimal medium (SD medium) for the

budding yeast *S. cerevisiae* (MMC) containing 1 g/l 5-FOA and incubated at 30 °C for approximately 14–21 days or until colonies emerged.

5-FOA-resistant colonies were isolated and transferred successively eight times to fresh medium with 5-FOA. The *pyrF* gene including 5′ and 3′ UTR sequence and the *pyrG* gene with 5′ UTR were respectively PCR amplified with PW1819 and PW1828 and PW1829 and PW1833, cloned into pCR2.1 (Invitrogen) and verified by DNA sequencing.

The WT *R. delemar* *pyrF* gene cloned in a construct similar to the *pyrF* urablaster described by Garcia and colleagues for *M. circinelloides* (Garcia et al., 2017) was used to complement the CDC-8219 *pyrF*-f2 mutant to prototrophy. The tandem repeat sequences surrounding the *pyrF* gene would facilitate an excision of the *pyrF* gene in theory, allowing recycling of the marker (Garcia et al., 2017). A 200-bp fragment from the 5′ UTR of the *R. delemar* *pyrF* gene was tandemly incorporated into the downstream, generating the *pyrF-dpl200* marker gene (Sup. Fig. S1). For complementation, spores were collected from five-day old cultures and incubated in liquid YPD at 30 °C with shaking (225 rpm) for about 4 h or until swelling was observed. Swelling spores were harvested, washed twice with 1.2 M sorbitol, incubated in 1.2 M sorbitol with 50 mM lithium acetate, 20 mM DTT at room temperature for 30 min. Spores were then washed once in 1.2 M sorbitol and plated on MMC-ura with 0.5 M sorbitol for biolistic transformation (Garcia et al., 2017). Biolistic transformation was similar to that for *C. neoformans* (Wang et al., 2004) with the exception that the gold particle beads were replaced with tungsten beads (0.7 µm, Bio-Rad).

4.3. Construction of plasmids for CRISPR-Cas9-mediated gene editing in *R. delemar*

pmCas9:tRNA-gRNA was recently utilized in the CRISPR-Cas9-mediated gene editing of the rice false smut fungus *U. virens* (Liang et al., 2018) and it has also been modified for gene editing in the basidiomycetous fungus *C. neoformans* (Wang, 2018). Similar to other CRISPR-Cas9 applications, the *CAS9* gene, fused with a nuclear localization signal from Simian virus 40 (SV40nls), is a codon-optimized version for expression in *M. oryzae* (Arazoe et al., 2014; Arazoe et al., 2013). There are two BsmBI restriction sites with an inverted orientation to prevent self-ligation engineered in the pmCas9-tRNA-gRNA plasmid allowing for the insertion of gRNA sequences for transcription. gRNA target sequences were identified from the *PyrF* sequence using the <http://www.flycrispr.molbio.wisc.edu/tools>. Two complementary 20-nt primers were designed, with the sense primer containing “ACCT” at the 5′ end, and the antisense primer containing “AAAC” at the 5′ end. The complementary primer pairs were phosphorylated with T4 polynucleotide kinase (NEB) to improve ligation efficiency and annealed by boiling for 5 min and gradually cooling down and then ligated into pmCas9:tRNA-gRNA at the BsmBI sites. The presence of gRNA insert was confirmed by DNA sequencing. DNA sequencing was performed at Eurofins Genomics.

A hygromycin resistant gene cassette was amplified from the plasmid pPDC-Cas9 with primers PW1916 and PW1917 and inserted in the *pyrF* gene at the AgeI site. Primers PW1823 and PW1827 were used to amplify the *pyrF::HYG* allele used as the donor DNA (Table 1).

4.4. Electroporation and transformant selection

Spores were prepared as described above for biolistic transformation. To improve transformation competency, 50 mM LiAc and 20 mM DTT (final concentrations) were added to spores and the mixture was incubated at room temperature for another 30 min. Spores were then washed twice with 1.2 M sorbitol and incubated on ice until use.

For electroporation, DNA was mixed with 50 µl of swollen spores ($\sim 1 \times 10^7$ cfu/ml) and the mixture was subjected to electroporation with the setting of time constant, 1100 V, and 5 ms. Spores were allowed to recover in liquid YPD with 0.5 M sorbitol for 90 min prior to plating onto MMC medium containing 2 g/l 5-FOA (with or without 200 µg/ml hygromycin). Colonies were transferred by removing a tiny agar block (1 mm³) containing 1–2 hyphal tips using a surgical scalpel and under a dissecting microscope and transferred to fresh plates. The process was repeated for at least 8 times prior to characterization.

5. Nucleic acid preparation and manipulation.

Strains were incubated in liquid MMC with 1 g/l 5-FOA (*pyrF*⁻) for 5–7 days and mycelia were collected. Mycelia were then ground into fine powders in liquid nitrogen and DNA was extracted following the standard protocol (Sambrook and Russell, 2001). For smaller scale DNA preparations, mycelia were collected in a small vial and fragmented by using a FastPrep bead beater (ThermoSavant) at the setting of 6 cycles of 40 s each. Cell lysates were recovered, extracted once each with phenol::chloroform:isoamyl alcohol reagent and then with chloroform. The DNA was precipitated with isopropanol.

5.1. Nucleus staining with DAPI

Hyphae were suspended in 1:1000 dilution of stock DAPI (5 mg/ml) and placed under a fluorescent microscope (Zeiss Imager.A2) for observation. For spores, they were first incubated in liquid YPD for 4 h for swelling and the swollen spores were then incubated in DAPI for 10 min and washed with distilled sterile water prior to the microscopic observation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Characterization of spontaneous *pyrF*- mutants of *Rhizopus delemar* FGSC-9543 and CDC-8219 strains.

(A) Typical appearance of spontaneous 5-FOA resistant colonies of CDC-8219 after incubation for 5 days at 30 °C. The typical *pyrF*- mutant is pointed by a white arrow. The colony pointed by the black arrow typically failed to retain 5-FOA resistance in subsequent culturing. (B) DNA sequencing of the 3.0-kb fragment amplified by primers PW1819 and PW1827 that contains the *pyrF* 5'-UTR and ORF. (C) Growth of the *R. delemar pyrF*- f2 mutant in comparison to wild type CDC-8219 and complemented TF1 and TF3 strains on potato dextrose agar (PDA), MMC –uracil, and MMC + 5-FOA (1 g/L). Incubation was carried out at 30°C for 1–5 days as indicated. (D) DNA sequencing of the 217-bp PCR fragment amplified with primers PW1972 and PW1945 (top chromatograms) and cloned products (lower chromatograms). (E) Primers PW2015 and PW2050 were used for PCR (30 cycles for the *pyrF*-dpl200 control and 35 cycles for the rest) to analyze complemented *pyrF*- mutant strains. A ~400-bp is expected for the *pyrF*- mutant and ~600-bp is expected for the integrated *pyrF*-dpl200 marker. Lane 1: *pyrF*-dpl200, lane 2: *pyrF*(91–92 TC), lane 3 and 4: complemented TF #1 and TF #3, respectively.

Fig. 2. Diagram of pmCas9:tRNA-gRNA for CRISPR-Cas9 in *R. delemar*.

Schematic representation of partial pmCas9-tRNA-gRNAF1 plasmid containing Cas9 and gRNA expression cassettes. A *Ustilaginoidea virens* Gln-tRNA sequence exhibiting Pol III promoter activity (indicated in dark blue ink) was used to drive the expression of gRNA. There are two inverted BsmBI/Esp3I restriction enzyme sites (in black ink) built into the sequence following the Gln-tRNA promoter. This construct is not cleavable by BsmBI/Esp3I once the annealed 20-nt gRNA plus 4 nt linker sequence (*pyrF1* is illustrated in light blue ink) is inserted.

Fig. 3. Occurrence of 5-FOA resistant colonies following CRISPR-Cas9 mediated electroporation.

Representative plates following transformation with pmCas9:tRNA-gRNA. pmCas9:tRNA-*pyrF1* and pmCas9:tRNA-*pyrF2* were electroporated into 50 μ l of swollen spores with the setting of time constant, 1100 V, and 5 ms. Spores were allowed to recover for 90 min before plating onto the selective MMC medium. Plates were incubated for 5–7 days or until colonies emerged. The *pyrF::HYG* fragment (~3-kb) obtained by PCR amplification with primers PW1823 and PW1827 was used as homologous donor DNA. Arrows point to the putative *pyrF*-transformants.

Fig. 4. CRISPR-Cas9 induces single point deletion in pmCas9-tRNA-*pyrF1* target site of the *pyrF* gene.

Sequencing chromatograms and corresponding sequence diagrams of CRISPR-Cas9 induced *pyrF*⁻ mutations in FGSC-9543 and CDC-8219 strains. Primer PW1972 and PW2050 were used to amplify the *pyrF*⁻ PCR fragments that were sequenced directly and sequenced after cloning into pCR2.1. The protospacer adjacent motif (PAM) sequences were shaded in light grey in both chromatograms and sequence diagrams. Arrows point to the position of single nucleotide deletions.

Table 1

Primer used in this study.

PW1819	5'-AGACTAGTTGATCCATCCATCCCCTTCTTATCC-3'
PW1823	5'-GGGTACCTCACGTACGGTCG-3'
PW1827	5'-TTATTGCTTGATACCATATTGTGC-3'
PW1828	5'-AGACTAGTTAGCTGCTGCTGCTGCTGCC-3'
PW1829	5'-AGACTAGTCGACATCGTCATTGAAGAGTGGC-3'
PW1833	5'-CTCTAGTCTATTTATTGTGTAAAGC-3'
PW1860	5'-CGGAGGGCGAAGAATCTCGTGC-3'
PW1861	5'-CCGTCAGGACATTGTTGGAGCCG-3'
PW1862	5'-GAGCGAGGCGATGTTCTGGGG-3'
PW1863	5'-CCATTTGTCTCAACTCCGGAGC-3'
PW1887	5'-AGACTAGTTAGCTGCTGCTGCTGCTGCCACTTACTTTCTT-3'
PW1916	5'-GAACCGGTGCGCAATTAACCTCACTAAAGG-3'
PW1917	5'-GAACCGGTCAGGGCTGGTGACGGAATTTTCATAGTCAAGC-3'
PW1945	5'-AAACTGTAGGCAGGACCGAAAAGC-3'
PW1947	5'-ACCTGCCAATGATGTCTTAAAGTT-3'
PW1948	5'-AAACAACTTTAAGACATCATTGGC-3'
PW1972	5'-GGGAATGAAGGAATATCAACGTGAATTTATCG-3'
PW2014	5'-ACCTGCGCTCTTGCCAGAACCATC-3'
PW2015	5'-AAACGATGGTTCTGGCAAGAGCGC-3'
PW2050	5'-TCAATGAATGCCAACCTCAA-3'