

Construction of a Recyclable Genetic Marker and Serial Gene Deletions in the Human Pathogenic Mucorales *Mucor circinelloides*

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ABSTRACT *Mucor circinelloides* is a human pathogen, biofuel producer, and model system that belongs to a basal fungal lineage; however, the genetics of this fungus are limited. In contrast to ascomycetes and basidiomycetes, basal fungal lineages have been understudied. This may be caused by a lack of attention given to these fungi, as well as limited tools for genetic analysis. Nonetheless, the importance of these fungi as pathogens and model systems has increased. *M. circinelloides* is one of a few genetically tractable organisms in the basal fungi, but it is far from a robust genetic system when compared to model fungi in the subkingdom Dikarya. One problem is the organism is resistant to drugs utilized to select for dominant markers in other fungal transformation systems. Thus, we developed a blaster recyclable marker system by using the *pyrG* gene (encoding an orotidine-5'-phosphate decarboxylase, ortholog of *URA3* in *Saccharomyces cerevisiae*). A 237-bp fragment downstream of the *pyrG* gene was tandemly incorporated into the upstream region of the gene, resulting in construction of a *pyrG-dpl237* marker. To test the functionality of the *pyrG-dpl237* marker, we disrupted the *carRP* gene that is involved in carotenoid synthesis in *pyrG*⁻ mutant background. The resulting *carRP::pyrG-dpl237* mutants exhibit a white colony phenotype due to lack of carotene, whereas wild type displays yellowish colonies. The *pyrG* marker was then successfully excised, generating *carRP-dpl237* on 5-FOA medium. The mutants became auxotrophic and required uridine for growth. We then disrupted the calcineurin B regulatory subunit *cnbR* gene in the *carRP::dpl237* strain, generating mutants with the alleles *carRP::dpl237* and *cnbR::pyrG*. These results demonstrate that the recyclable marker system is fully functional, and therefore the *pyrG-dpl237* marker can be used for sequential gene deletions in *M. circinelloides*.

KEYWORDS

Mucor
circinelloides
URA blaster
pyrG blaster
recyclable
genetic marker
mucormycosis

Mucor circinelloides is a basal fungus belonging to the phylum Mucoromycota (Spatafora *et al.* 2016). *M. circinelloides* is one of the etiological agents of mucormycosis. Mucormycosis is an opportunistic fungal infection recently recognized as an emerging infectious disease (Kauffman

2004; Brown 2005; Chayakulkeeree *et al.* 2006; Lanternier *et al.* 2012a). Fungi in the order Mucorales are the causal agents of mucormycosis, and include *Mucor* spp., *Rhizopus* spp., *Lichtheimia* (previously *Absidia*) spp., *Apophysomyces* spp., *Cunninghamella* spp., *Rhizomucor* spp., and others (Chayakulkeeree *et al.* 2006; Ibrahim and Spellberg 2006; Neblett Fanfair *et al.* 2012). Recent data indicates a significant increase in mucormycosis due to a rising number of immunocompromised patients with conditions such as diabetes, HIV/AIDS, hematologic malignancies, hematopoietic stem cell/solid organ transplantation, or trauma (Ribes *et al.* 2000; Marr *et al.* 2002; Kontoyannis *et al.* 2005; Roden *et al.* 2005; Spellberg *et al.* 2005). Mucormycosis is the second most common mold infection among patients with hematological malignancies and transplants, and is associated with high mortality rates of ~50% for all mucormycosis infections and >90% in disseminated infections (Ribes *et al.* 2000; Roden *et al.* 2005; Lanternier *et al.* 2012b). Even after recovery, patients often suffer from

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permanent disfiguration in the affected areas due to surgical debridement as a frequent treatment modality (Kwon-Chung and Bennet 1992; Kontoyiannis *et al.* 2005; Roden *et al.* 2005; Spellberg *et al.* 2005; Kontoyiannis and Lewis 2006).

M. circinelloides has served as a system to study virulence and pathogenesis of mucormycosis in animal host models. For example, various *Mucor* isolates produce spores of different sizes, and the size of spores contributes to virulence as larger spores are more virulent than smaller spores (Li *et al.* 2011). The difference in virulence may be due to a difference in the interactions of larger and smaller spores with macrophages whereby larger spores germinate inside macrophages, and smaller spores remain dormant inside macrophages (Li *et al.* 2011). The morphogenesis of *M. circinelloides* also contributes to virulence, in which the filamentous form of the fungus is more virulent than the yeast form (Lee *et al.* 2013). In addition, the different morphogenic states (spores/hyphae vs. yeast) result in different host-pathogen interactions (Lee *et al.* 2015). For example, *Mucor* spores arrest or delay phagosome maturation upon phagocytosis by macrophages, whereas *Mucor* yeast do not. Cytokine responses from immune cells differ between *Mucor* spores/hyphae and yeast. A recent study found that a phospholipase D and myosin V family protein are required for full virulence in *Mucor* through an RNAi-based genome screen (Trieu *et al.* 2017).

M. circinelloides is also able to contaminate and spoil foods (Lazar *et al.* 2014; Lee *et al.* 2014). *M. circinelloides* is known to produce the toxin 3-nitropropionic acid (3NP) (Hollmann *et al.* 2008), which makes this fungus a potential cause of food poisoning. 3NP inhibits mitochondrial succinate dehydrogenase, causing acute brain injury, methemoglobinemia, dystonia after ingestion, and symptoms similar to Huntington's disease (Alston *et al.* 1977; Gabrielson *et al.* 2001; Brouillet *et al.* 2005). Interestingly, 3NP was responsible for 217 sugarcane poisoning outbreaks in China during the period 1972–1989, which involved 884 patients with 88 mortalities [reviewed in Magan and Olsen (2004)]. Therefore, it is apparent that *Mucor* poses a threat to public health.

M. circinelloides also serves as a model to study light sensing in fungi. Asexual sporulation is triggered by light. Blue light activates carotenoid biosynthesis and sporangiophore phototropism (Navarro *et al.* 1995; Silva *et al.* 2006). *Mucor* also encodes homologs of the *Neurospora crassa* white collar-1 (WC-1), which forms the white collar complex with other factors that entrain the circadian clock (Liu and Bell-Pedersen 2006). The functions of the three *wc-1* genes have been elucidated in *Mucor*, in that they are all involved in light response although each of them has distinct light transduction pathways (Silva *et al.* 2006, 2008; Navarro *et al.* 2013).

M. circinelloides also has a conserved and active RNAi pathway that governs the expression of nonintegrated transgenes (Nicolás *et al.* 2003), and therefore serves as a model to study RNA silencing in fungi. Genetic analysis demonstrated that Dicer, Argonaute, and RNA-dependent RNA polymerase play major roles in this RNA silencing, and these three components are conserved in *M. circinelloides* (de Haro *et al.* 2009; Calo *et al.* 2012; Billmyre *et al.* 2013; Cervantes *et al.* 2013). Interestingly, *M. circinelloides* exhibits a novel mechanism of drug resistance evoked by RNAi-mediated silencing (Calo *et al.* 2014). In the presence of the calcineurin inhibitor FK506, the fungus can silence the target gene of FK506, which encodes FKBP12, to become drug resistant. However, without selective pressure, the fungus will eventually deactivate the silencing mechanism and become drug sensitive in an epigenetic RNAi mediated process (Calo *et al.* 2014). A recent study found that the noncanonical RdRP-dependent/Dicer-independent RNAi pathway indeed inhibits the canonical RNAi-mediated drug resistant

pathways, suggesting a balance between two RNAi pathways in *M. circinelloides* (Calo *et al.* 2017).

The recent oil crisis and environmental conservation initiatives have prompted innovations in the production of biofuel. Research has focused on oilseed crops and plant-based carbohydrates that can be converted into fatty acid esters by microorganisms (Kalscheuer *et al.* 2006; Steen *et al.* 2010). An additional important biofuel source is lipid-accumulating microorganisms, such as oleaginous fungi and algae (Kosa and Ragauskas 2011). *M. circinelloides* is an oleaginous microorganism that accumulates lipids as a storage source (Vicente *et al.* 2009; Kosa and Ragauskas 2011; Rodríguez-Frómata *et al.* 2013) and produces a high level of linoleic acid, which served as a basis for the first commercial microbial lipid (Ratledge 2004). Biodiesel produced by *M. circinelloides* satisfies the specifications set by American and most European standards (Vicente *et al.* 2009).

M. circinelloides is clearly a prominent organism in many aspects of biology. Unfortunately, the genetics of this fungus are limited. Resistance to drugs utilized to select for dominant markers in other fungal transformations, including neomycin, nourseothricin, hygromycin, zeomycin, carboxin, and glufosinate, is common (data not shown). This precludes the use of these common dominant drug resistance markers for *Mucor*. Only two auxotrophic markers (*leuA*⁻ and *pyrG*⁻) in one strain (Nicolas *et al.* 2007) and one auxotrophic marker (*Met*⁻) in another strain (Anaya and Roncero 1991) are available in the background of the sequenced isolates (<http://genome.jgi-psf.org/Mucci2/Mucci2.home.html>), which significantly limits the ability to disrupt multiple genes for genetic analysis.

In this study, we developed a recyclable *pyrG* blaster marker for multiple gene deletions in *M. circinelloides* that is analogous to the *URA3* blaster marker for *Candida albicans* (Wilson *et al.* 2000). A 237-bp fragment of the 3'-downstream of the *pyrG* gene was tandemly incorporated in the 5'-upstream of the *pyrG* to generate a *pyrG-dpl237* (237 bp-*pyrG*-237 bp) marker. The marker was used to successfully disrupt the *carRP* gene to generate an albino mutant (*carRPΔ::pyrG-dpl237*). Spontaneous excision of the *pyrG* marker in the *carRPΔ::pyrG-dpl237* allele occurred; resulting in generation of *carRPΔ::dpl237* mutants. Additionally, the *cnbR* gene was disrupted by using *pyrG* gene as a marker, demonstrating the *pyrG* blaster marker is fully functional and can be used for multiple gene disruption.

MATERIALS AND METHODS

Fungal strains and growth conditions

The strains and plasmids used in this study are listed in Table 1. For spore production, *M. circinelloides* strains were grown on yeast peptone glucose agar (YPG, 3 g/liter yeast extract, 10 g/liter peptone, 20 g/liter glucose, 2% agar, pH 4.5) media at 26° in the light for 4 d. To collect spores, deionized sterile water was added to the plates and the spores were gently scraped with a cell spreader. For further experiments, the number of spores was calculated with the automated cell counter TC20 (Bio-Rad) or a hemocytometer. YPD agar was used to compare the phenotypes of yeast-locked *cnbRΔ* mutants with wild type. For transformation, minimal media with casamino acids (MMC; pH 3.2, 10 g casamino acids, 0.5 g yeast nitrogen base without amino acids and ammonium sulfate, 20 g glucose, 1 mg niacin, 1 mg thiamine, and 15 g agar in 1 liter dH₂O) media containing 0.5 M sorbitol was used.

M. circinelloides spores are multinucleate, so vegetative selection was required to isolate homokaryotic transformants. The initial heterokaryotic transformants were subjected to vegetative rounds of growth

■ **Table 1** List of strains and plasmids used in this study

	Name	Genotypes or Characteristics	Reference
<i>M. circinelloides</i> strains	MU402	<i>leuA</i> ⁻ <i>pyrG</i> ⁻	Nicolas <i>et al.</i> (2007)
	MSL7	<i>leuA</i> ⁻ <i>pyrG</i> ⁻ <i>cnbRΔ::pyrG</i>	Lee <i>et al.</i> (2013)
	MSL27	<i>leuA</i> ⁻ <i>pyrG</i> ⁻ <i>carRPΔ::pyrG-dpl237</i>	This study
	MSL28	<i>leuA</i> ⁻ <i>pyrG</i> ⁻ <i>carRPΔ::dpl237</i>	This study
	MSL29	<i>leuA</i> ⁻ <i>pyrG</i> ⁻ <i>carRPΔ::dpl237</i>	This study
	MSL30	<i>leuA</i> ⁻ <i>pyrG</i> ⁻ <i>carRPΔ::dpl237</i>	This study
	MAG1	<i>leuA</i> ⁻ <i>pyrG</i> ⁻ <i>carRPΔ::dpl237 cnbRΔ::pyrG</i>	This study
	MAG2	<i>leuA</i> ⁻ <i>pyrG</i> ⁻ <i>carRPΔ::dpl237 cnbRΔ::pyrG</i>	This study
	Plasmids	pCR21-TOPO	Ampicillin ^R Kanamycin ^R
pTOPO-pyrG		2002-bp <i>pyrG</i> fragment cloned into pCR21-TOPO	Lee <i>et al.</i> (2013)
pCnbR-KO		<i>cnbR</i> disruption construct cloned into pCR21-TOPO	Lee <i>et al.</i> (2013)
pSL13		<i>pyrG</i> blaster marker in pCR21-TOPO	This study

on MMC media to generate homokaryotic transformants. If necessary, MMC (pH 4.5) media was supplemented with 0.61 g/liter of uridine and 0.56 g/liter uracil. To select mutants that spontaneously excise the *pyrG* gene, 5-FOA (2.5 mg/ml) was supplemented to MMC (pH 4.5) media.

Development of a recyclable *pyrG* marker

A 2002-bp DNA fragment containing the *pyrG* gene was amplified with M13 forward and reverse primers from the vector pTOPO-pyrG that we previously constructed (Lee *et al.* 2013). This plasmid was used as an initial plasmid to construct a 237 bp-*pyrG*-237 bp (or *pyrG-dpl237*) allele. The 237-bp DNA fragment located downstream of the *pyrG* gene was amplified with the primers SCL635 and SCL636 (Table 2), which contain overhanging *Xba*I or *Not*I restriction enzyme recognition sites on either side. The amplified 237-bp fragment was cloned into pPyrG-TOPO digested with *Xba*I and *Not*I, generating the pSL13 (Table 1), which harbors a DNA cassette with 237-bp direct repeats on both sides of the *pyrG* gene.

Disruption of genes

To disrupt the *carRP* gene, we constructed a disruption cassette containing the *pyrG* blaster marker flanked by ~1 kb of 5' and 3' of the up- and downstream regions of the *carRP* gene via overlap PCR. The 5' region was amplified with primers SCL639 and SCL640, and the 3' region was amplified with primers SCL641 and SCL642. Genomic DNA of strain MU402 served as template. The *pyrG* blaster (*pyrG-dpl237*) marker was amplified with M13 forward and reverse primers. The three fragments were then subjected to an overlap PCR with nested primers SCL643 and SCL644, to generate a disruption allele. A mixture of 75 ng of each fragment was used as template. To disrupt the *cnbR* gene in the *carRPΔ* mutant background, the *cnbR* disruption cassette was amplified from plasmid pCnbR-KO (Lee *et al.* 2013) via PCR using a pair of primers, SCL286 and SCL287, with the Thermo Scientific DreamTaq DNA polymerase. The *cnbR* disruption construct was introduced into the MSL29 (*leuA*⁻ *pyrG*⁻ *carRPΔ::dpl237*) strain via electroporation as described previously (Lee *et al.* 2013). In brief, 10⁸ germinated spores were protoplasted in the presence of chitosanase (US Biological) and lysing enzyme (Sigma). The protoplasts were resuspended in 0.5 M sorbitol solution and mixed with 3 μg of the disruption cassette DNA. Electroporation was performed in 0.2-cm cuvettes (pulse at 0.8 kV, 25 μF, 400 ohm) and the protoplasts were spread onto selective MMC (pH 3.2) media supplemented with 0.5 M sorbitol. From 16 independent transformations, we obtained five mutants that exhibit the yeast-

locked phenotype. Two independently obtained mutants, MAG1 and MAG2, were used in this study (Table 1).

Southern blotting analysis

Southern blotting was performed to ensure the absence of the *carRP* gene, the presence of *carRP::pyrG-dpl237*, and the excision of the *pyrG* gene from the *carRP::pyrG-dpl237* allele. Genomic DNA (30 μg) of the wild type and *carRP::pyrG-dpl237* and *carRP::dpl237* mutants was digested using either *Eco*RI or *Bcl*I enzyme, or both. The digested genomic DNA fragments were separated in a 1% agarose gel. The gel was then rinsed in deionized distilled water and placed in a denaturing solution (1.5 M NaCl, 0.5 M NaOH). The gel was then rinsed again with deionized distilled water and placed in a neutralization solution [1.5 M NaCl, 0.5 M Tris-HCl (pH 7.2), 1 mM EDTA]. Capillary transfer of DNA into nylon membrane was performed for 18 hr. The membrane was then placed in a UV cross-linking chamber (Spectrolinker XL-1000 UV cross-linker with an autoexposure setting; Spectronics Co.) to cross-link DNA to the membrane. The probe was generated by PCR with primers SL1 and SL2. The probe was then labeled with biotin by using the Thermo Scientific Biotin DecaLabel DNA labeling Kit following the manufacturer's instruction. The Thermo Scientific Biotin Chromogenic Detection Kit was used to detect the labeled probes on the membrane following the manufacturer's instruction.

Excision of the *pyrG* gene from the *pyrG* blaster marker

Spores of the MSL27 (*carRPΔ::pyrG-dpl237*) strain were placed on MMC media containing 5-FOA (2.5 mg/ml), uridine (0.61 mg/ml), and uracil (0.56 mg/ml), to select mutants that still produced white colonies but required uridine to grow. The candidate mutants were passaged on MMC media containing uridine, uracil, and 5-FOA. Excision of the *pyrG* gene was verified by PCR and Southern blotting.

Data availability

All the strains and plasmids used in this study are available upon request. The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

RESULTS AND DISCUSSION

Generation of *pyrG* blaster marker and disruption of the *carRP* gene

We developed a *pyrG* blaster recyclable marker system by using the *pyrG* gene (encoding orotidine-5'-phosphate decarboxylase, ortholog of *URA3* in *Saccharomyces cerevisiae*). To construct a *pyrG* blaster

■ Table 2 Primers used in this study

Name	5' → 3' Sequence	Remark
SCL639	CACCTTAGCAGCAGCAATCA	Primer for <i>carRP</i> disruption, 5' region
SCL640	ACTGGCCGTCGTTTTACCGGTGTGGAGAAAGAGGTGT	Primer for <i>carRP</i> disruption, 5' region
SCL641	GCATAGCTGTTTCCTGCACTATCCCAGCAGAGCACACA	Primer for <i>carRP</i> disruption, 3' region
SCL642	GCCATTTGTGTCTTGGGTTT	Primer for <i>carRP</i> disruption, 3' region
SCL643	ATGGCCATCTTCTTGGTCTT	Nested forward primer for <i>carRP</i> disruption
SCL644	GCTTCTTTCTGGCATGTGT	Nested reverse primer for <i>carRP</i> disruption
SCL649	GGGAGGCAACAGATTGTGAT	5' outside forward of <i>carRP</i> disruption confirmation
SCL650	TCTTTTTGCTGTTGCTGTGC	3' outside reverse of <i>carRP</i> disruption confirmation
SCL566	ACCCACTCACTTCCATTCCG	Primer for <i>pyrG</i> to determine insertion
SCL567	TGCTTTTGTGGCTGAGATG	Primer for <i>pyrG</i> to determine insertion
SCL635	TCTAGA GGGAGTCAAGGCAGGTCTTT	<i>pyrG</i> blaster 237-bp fragment with <i>XbaI</i> overhang
SCL636	GCGGCCGCTGATAACTGATGCGCTGCAT	<i>pyrG</i> blaster 237-bp fragment with <i>NotI</i> overhang
SCL286	CGGCAAGTACTGTGTCTCA	Forward primer for <i>cnbR</i> disruption
SCL287	ATGGCAAAGTCGAAGAGGAA	Reverse primer for <i>cnbR</i> disruption
SL1	TCCACCACAGTGACCTTGAA	Forward primer for Southern blotting probe for <i>carRP</i>
SL2	GGGGAGTGATAGGGCAGAAT	Reverse primer for Southern blotting probe for <i>carRP</i>
JOHE22226	TAAGGTCGATGTTGCCACTG	Forward primer for 5' outside of <i>cnbR</i> disruption confirmation
JOHE22231	CGAAAAACAACAACGCATTG	Reverse primer for 3' outside of <i>cnbR</i> disruption confirmation

DNA cassette that can be easily amplified by PCR, we adapted a 237-bp repeat as previously described in *C. albicans* (Wilson *et al.* 2000). In our previous study, we generated the pTOPO-*pyrG* plasmid harboring a 2002-bp DNA fragment containing the *pyrG* gene (Lee *et al.* 2013) (Figure 1A, top). The plasmid was used as an initial plasmid to construct a 237 bp-*pyrG*-237 bp (or *pyrG-dpl237*) allele. The 237-bp DNA fragment located at the downstream of the *pyrG* gene was amplified with primers that contain overhanging *XbaI* or *NotI* restriction enzyme recognition sites on each side. The amplified 237-bp fragment was cloned into pTOPO-*pyrG* digested with *XbaI* and *NotI*, generating the pSL13 (Figure 1A, bottom), which harbors a DNA cassette with 237-bp direct repeats on both sides of the *pyrG* gene. The tandem-repeated 237 bp on both side of the *pyrG* gene enhances a spontaneous homologous recombination between them, resulting in excision of the *pyrG* gene. The marker gene therefore can be used for another gene disruption.

To test the functionality of the *pyrG* blaster marker, we targeted the *carRP* gene, which encodes a protein with dual enzyme activities (lycopen cyclase and phytoene synthase) that are involved in carotenoid synthesis in *M. circinelloides* (Velayos *et al.* 1997) (Figure 1B). Complete loss of the *carRP* gene resulted in white colony formation due to the lack of accumulation of carotenoids, whereas the wild type displayed yellowish colonies. This characteristic of the *carRP* mutants enabled us to screen homokaryotic homologous recombinant transformants by evaluating the colony color (Rodríguez-Frómata *et al.* 2013). We obtained seven transformants from eight independent transformations. Progeny from two transformants displayed both white and yellow colonies. The white and yellow mixed progeny indicate that the nuclei of the transformants are heterogeneous, containing nuclei with *carRPΔ* and wild-type *carRP* alleles. *Mucor* spores are multinucleate and their hyphae are coenocytic (aseptate), so cycles of vegetative passage under selective conditions are therefore required to enrich recombinant nuclei. The transformant MSL27 (*carRPΔ::pyrG-dpl237*) stably produced exclusively white colony progeny, and this strain was used to test the recyclable *pyrG* marker.

We further validated that spores of the MSL27 strain contain only the *carRPΔ* allele, and not the wild-type allele. The primers SCL649 (P1) and SCL650 (P2) outside of the disruption cassette were used to test the genotype of the progeny. A 4020-bp PCR fragment was produced from the wild-type *carRP* allele, and a 4649-bp fragment was produced from

the *carRPΔ* allele, which confirms that the MSL27 strain does not contain the wild-type *carRP* allele (Figure 2). The disruption of the *carRP* gene was further verified by a Southern blotting analysis (Figure 3). A single digestion with *BclI* and a double digestion with *BclI* and *EcoRI* were performed. The probe for the 5' upstream of the *carRP* gene recognized 5011-bp fragment when the wild-type genomic DNA was digested with *BclI* and 1322-bp fragment when double-digested with *EcoRI* and *BclI*. On the other hand, the replacement of the *carRP* gene with *pyrG-dpl237* introduced additional *EcoRI* and *BclI* recognition sites; the probe then recognized 2501-bp fragment when the genomic DNA of MSL27 (*carRPΔ::pyrG-dpl237*) was single-digested with *BclI*, and an 872-bp fragment when double-digested with *BclI* and *EcoRI*.

Excision of *pyrG* gene in the *carRPΔ::pyrG-dpl237* mutant

To obtain spontaneous mutants that excised the *pyrG* marker, spores of the MSL27 (*carRPΔ::pyrG-dpl237*) strain were placed on MMC media containing 5-FOA (2.5 mg/ml), uridine (0.61 mg/ml), and uracil (0.56 mg/ml), to select mutants that still produced white colonies but required uridine to grow. The mutants that grow in the presence of 5-FOA are likely homokaryotic because resistance to 5-FOA is recessive. Two candidates (MSL28 and MSL29) were obtained (5-FOA resistance frequency at 5.2 ± 1.3 progeny/10⁵ spores inoculated) and the absence of the *pyrG* marker gene was confirmed by PCR with the primers SCL649 (P1) and SCL650 (P2), in which a 2624-bp fragment was produced after excision of the *pyrG* gene, instead of the 4649-bp fragment from the *carRPΔ::pyrG-dpl237* allele (Figure 2). We confirmed that the two *carRPΔ::dpl237* strains, MSL28 and MSL29, produce white colonies (Figure 2). We also confirmed that the MSL28 and MSL29 (*carRPΔ::dpl237*) require uridine to grow on MMC media due to the excision of the functional *pyrG* gene. The excision of the *pyrG* gene was further verified by a Southern blotting analysis (Figure 3). When genomic DNAs of the two *carRPΔ::dpl237* mutants were single-digested with *BclI*, the probe recognized a 3539-bp fragment, whereas in wild type and *carRPΔ::pyrG-dpl237*, the probe recognized a 5011-bp or a 2501-bp fragment, respectively. When double-digested with *BclI* and *EcoRI*, the probe recognized an 870-bp fragment in the *carRPΔ::dpl237* mutants, whereas it recognized a 1322-bp or an 872-bp

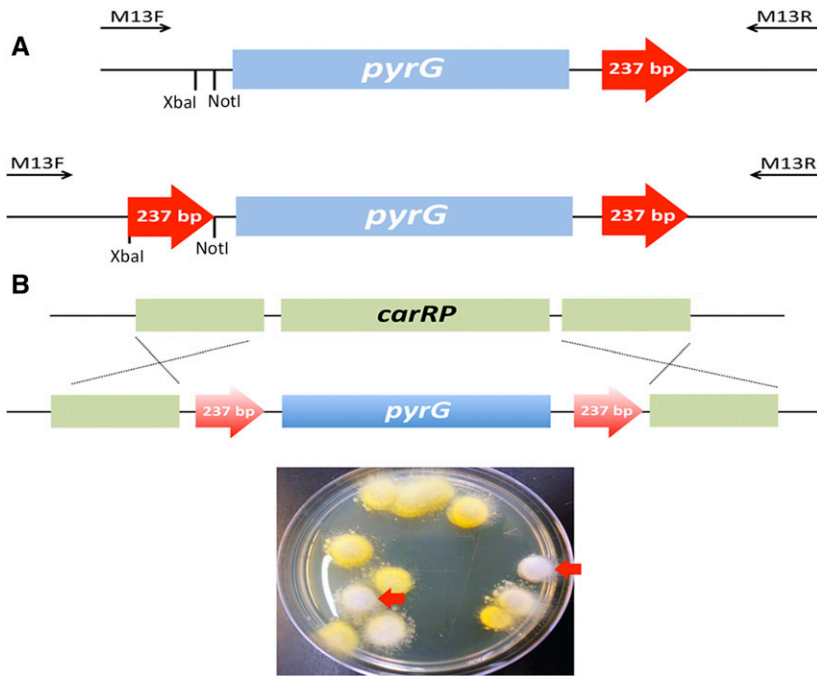


Figure 1 Development of a recyclable *pyrG* marker in *M. circinelloides*. (A) Construction of a *pyrG* blaster marker with repeats of 237 bp on 5' and 3' end. The short repeat (237 bp) in the 3' end was tandemly incorporated to enhance the excision of the marker gene. The resulting *pyrG* blaster marker can be amplified by PCR with M13 forward and reverse primers. (B) Homologous replacement of the *carRP* gene with the *pyrG* blaster marker. The loss of the *carRP* gene results in a failure to produce carotenoids in *M. circinelloides*. Therefore, the *carRP*Δ mutants display white colonies as indicated by the red arrows on the plate, whereas wild-type cells form yellow colonies. Although the target gene is disrupted, only a portion of the nuclei contains the *carRP*Δ allele because the spores are multinucleated with aseptate hyphae. Transformants that stably form white colonies were selected to obtain the *carRP*Δ mutant with a homozygous karyotype. Gene size is not to scale.

fragment in wild type or the *carRP*Δ::*pyrG-dpl237* mutant, respectively (Figure 3). These results confirm the *pyrG* gene was successfully excised from the locus and the *pyrG* gene can be used to disrupt another gene.

Disruption of the *cnbR* gene in a *carRP*Δ::*dpl237* mutant

We proceeded to disrupt the calcineurin B regulatory subunit *cnbR* gene in the *carRP*Δ::*dpl237* strain to verify that the recyclable marker system was fully functional. In our previous study, we found that the disruption of *cnbR* gene resulted in a yeast-locked phenotype, which makes

screening for homologous recombinant progeny straightforward. The MSL29 strain (*carRP*Δ::*dpl237*) was subject to transformation to disrupt the *cnbR* gene. This resulted in 75 transformants from 16 independent transformations. We screened the transformants by PCR with the *cnbR* deletion-specific primers JOHE22226 and SCL566 (Table 2, P1 and P2 in Supplemental Material, Figure S1, respectively) as previously described (Lee *et al.* 2013). Five transformants were found to carry an allele with *cnbR*::*pyrG* (data not shown). Homokaryotic *cnbR*::*pyrG* mutants exhibit a yeast-locked phenotype (Lee *et al.* 2013). After two

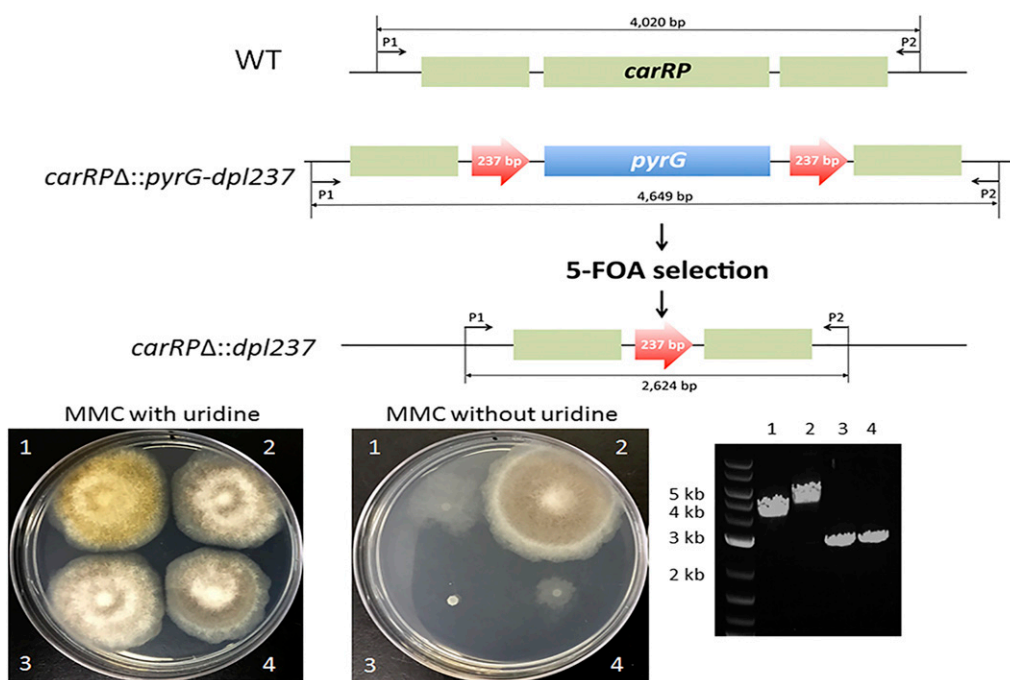


Figure 2 Confirmation of excision of the *pyrG* marker. The MSL27 (*carRP*Δ::*pyrG-dpl237*) strain was spot-inoculated on MMC media containing 5-FOA. After 3 d of incubation, spontaneous resistant mutants emerged forming a sector from colony (data not shown). The 5-FOA resistant mutants went through a vegetative cycle on MMC media containing 5-FOA. The obtained spores were subject to PCR analysis and auxotrophy tests. The excision of the *pyrG* marker from the *carRP*Δ::*pyrG-dpl237* allele was confirmed by PCR (see text in the section of Excision of *pyrG* gene). The strains with the *pyrG* marker excised did not grow on media without uridine. Two independent *carRP*Δ::*dpl237* strains were obtained. (1) MU402 (WT in *carRP* allele and *pyrG*⁻); (2) MSL27 (*carRP*Δ::*pyrG-dpl237*); (3) MSL28 (*carRP*Δ::*dpl237*); (4). MSL29 (*carRP*Δ::*dpl237*). Gene size is not to scale.

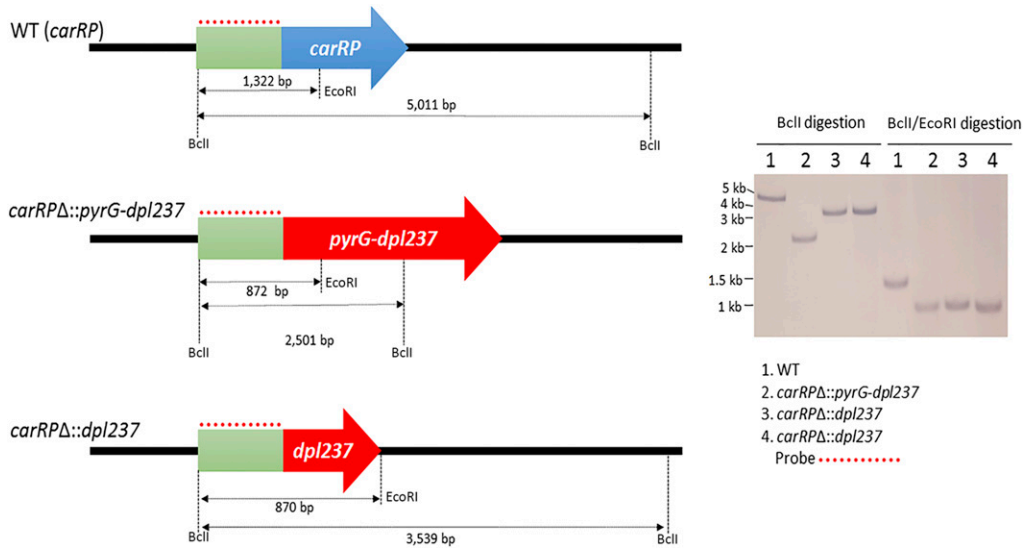


Figure 3 Southern blot analysis confirms the deletion of the *carRP* gene and excision of the *pyrG* marker gene. Southern blotting was performed to ensure the deletion of the *carRP* gene and excision of the *pyrG* marker. Genomic DNA of the wild-type strain was digested with *BclI* and produced a 5011-bp fragment that was recognized by the probe. A double digestion with *BclI* and *EcoRI* resulted in a 1322-bp fragment that was recognized by the probe. The digestion of the genomic DNA of the *carRP*Δ::*pyrG-dpl237* mutant with *BclI* produced a 2501-bp fragment that was recognized by the probe. The double digestion with the enzymes *BclI* and *EcoRI* produced an

872-bp fragment recognized by the probe. Finally, the *carRP*Δ::*dpl237* mutant underwent the same digestions, where a 3539-bp fragment was produced after *BclI* enzyme digestion, and an 870-bp fragment was produced after *BclI* and *EcoRI* double digestion. Both fragments were detected during our hybridization procedure by our probe. Gene size is not to scale.

rounds of vegetative passage on selective media, all five of them produced yeast-locked colonies along with wild-type colonies that were heterokaryotic containing nuclei either with *cnbR* or *cnbR::pyrG*. The yeast-locked colonies were selected and it was confirmed that they carry *cnbR::pyrG* and *carRP::dpl237* by PCR (Figure S1). A disruption in the *cnbR* gene in the *carRP*Δ::*dpl237* strain resulted in progeny that were both yeast locked and white in color (Figure 4). The results verified that the recyclable *pyrG* blaster marker is fully functional and can be used for a series of gene deletions in *M. circinelloides*.

Conclusions

Compared to ascomycetes and basidiomycetes, basal fungal lineages have been understudied. This may be caused by a deficit in attention

given to these fungi, as well as fastidious genetic analysis. However, the importance of these fungi as pathogens and model systems has increased. *M. circinelloides* is one of very few genetically amenable organisms belonging to the basal fungi, but it is far from a robust genetic system when compared to model fungi in the Dikarya. *M. circinelloides* is a human fungal pathogen of significant public health concern. The lack of robust genetic tools presents an obstacle for investigating the biology of this pathogen with an aim toward the development of effective antifungal drugs. This study generates the facile genetic tool, recyclable *pyrG* marker, which will drive fundamentally important biomedical research on this pathogen, while providing a broader and sustainable impact on the field by enabling detailed genetic analyses of related Mucoralean fungi of relevance to human health.

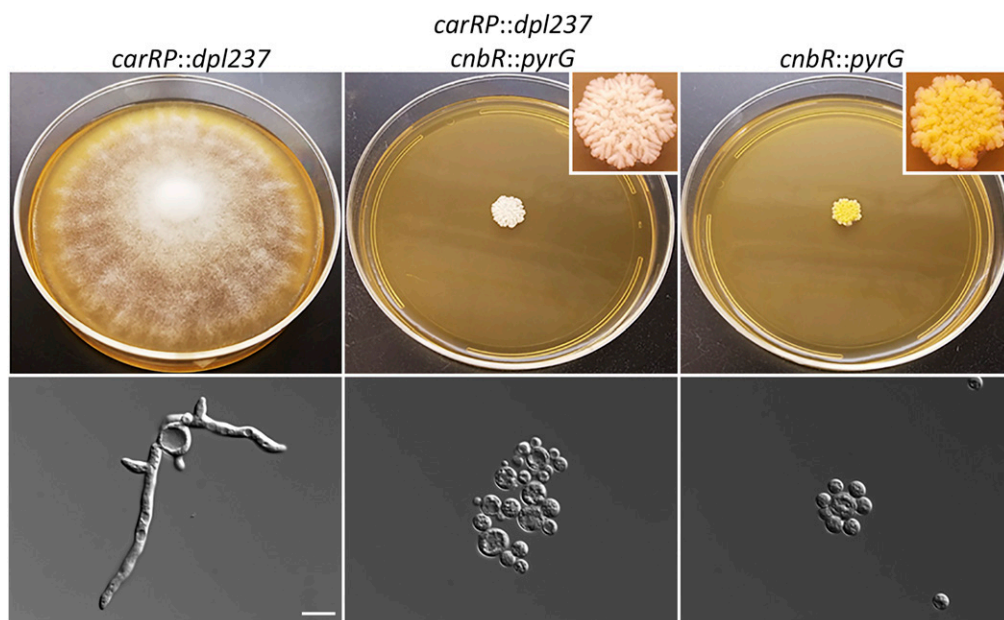


Figure 4 Disruption of the *cnbR* gene in the *carRP*Δ::*dpl237* mutant and phenotypes of the double mutants. The *carRP*Δ::*dpl237* strain, MSL28, produces white colonies and requires uridine to grow. The *cnbR*Δ::*pyrG* strain, MSL7, produces a yeast-locked colony while still retaining the function of the *carRP* gene, and thus produces yellow yeast-locked progeny. The *carRP*Δ::*dpl237* *cnbR*Δ::*pyrG* strain, MAG1, exhibits a yeast-locked phenotype and produces white colonies due to the disruption in both *carRP* and *cnbR* genes. Microscopic images were also taken to confirm the phenotype of each strain. Bar, 10 μm.

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