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Supplemental information

Stable and reproducible homologous recombination enables CRISPR-based engineering in the fungus *Rhizopus microsporus*

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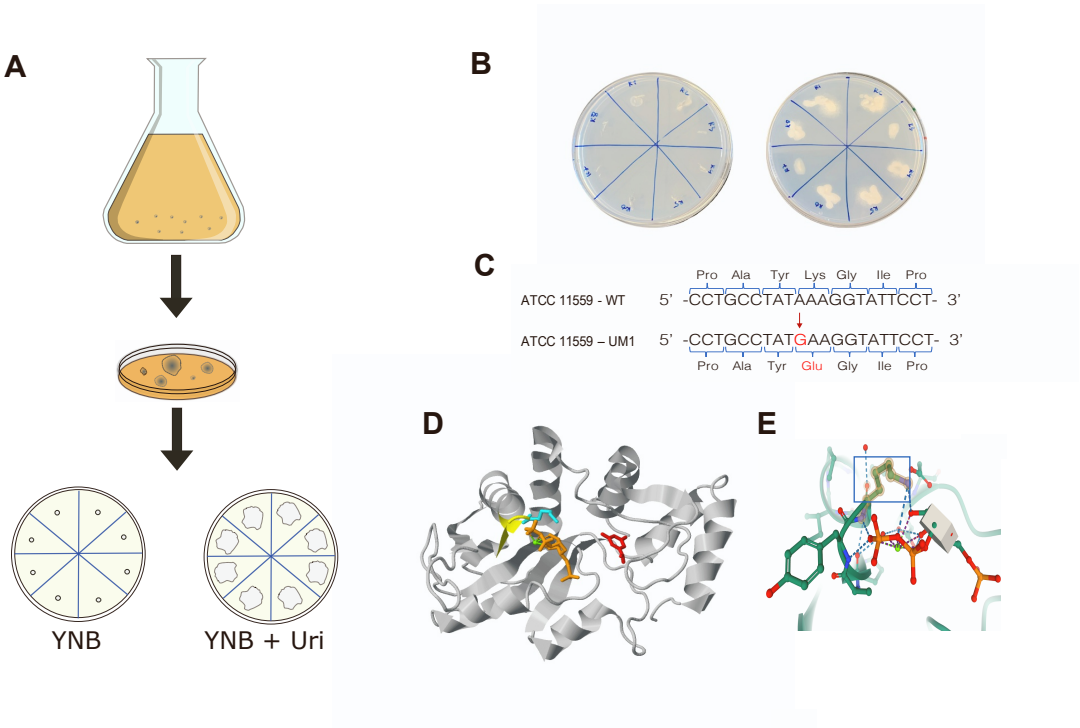

Figure S1. Generation of the uridine auxotrophic strain UM1 and mutation characterization, Related to STAR Methods. (**A**) Schematic representation of the workflow followed to isolate uracil auxotrophic strains. (**B**) 5-FOA resistant isolates R1-R8 after 24 hours of growth on YNB with (right) and without uridine (left). (**C**) Nucleotide sequences and encoded amino acids of *pyrF* gene sequence (+207-+227) that differed between the *R. microsporus* wild-type strain ATCC 11559 and the UM1 strain. The nucleotide mutation and the amino acid change found in the UM1 are highlighted in red. (**D**) Orotate phosphoribosyltransferase (PyrF) structure of *S. typhimurium*. The active site is colored in yellow (71-74), the Lys73 residue is colored in blue, the orotic acid is colored in red, and the PRPP is colored in orange. (**E**) Representation of the Lys73 residue highlighted in yellow (inside a blue square) and its interactions with PRPP and other residues.

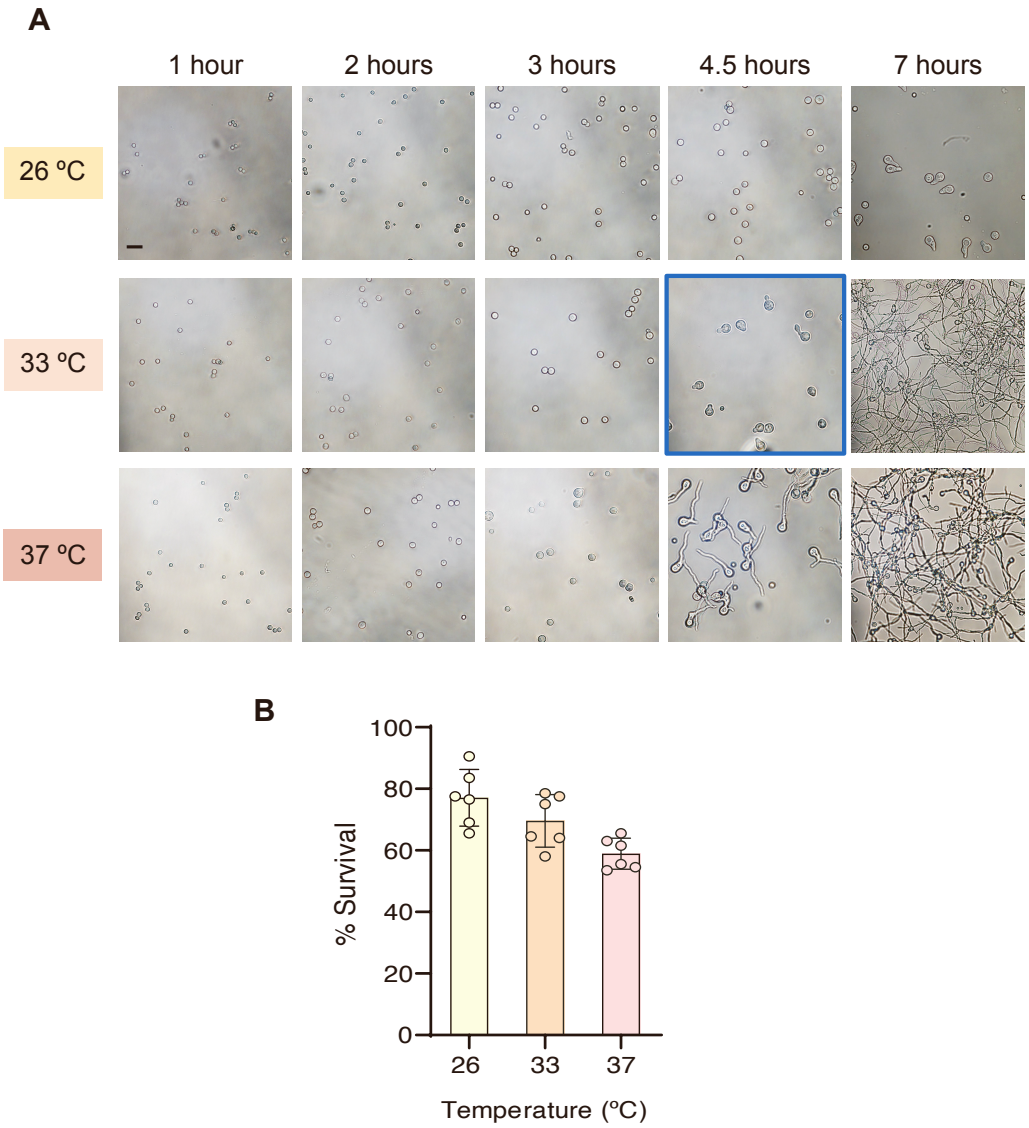

Figure S2. Effect of temperature in germination and protoplast viability, Related to STAR Methods. (**A**) Germination at 26 ºC (top), 33 ºC (middle), and 37 ºC (above) of the UM1 strain in rich YPG media supplemented with uridine. Images were taken (from left to right) at 1, 2, 3, 4.5, and 7 hours. The optimal germination stage for protoplast generation (33 ºC and 4.5 hours) is framed in blue. Scale bar = 20 µm. (**B**) Spores of the UM1 strain were germinated at 26 ºC, 33 ºC, and 37 ºC. After cell wall digestion, two hundred protoplasts were plated in minimal media (MMC) supplemented with uridine. Each point corresponds to the calculated percent survival of each plate (6 plates/condition).

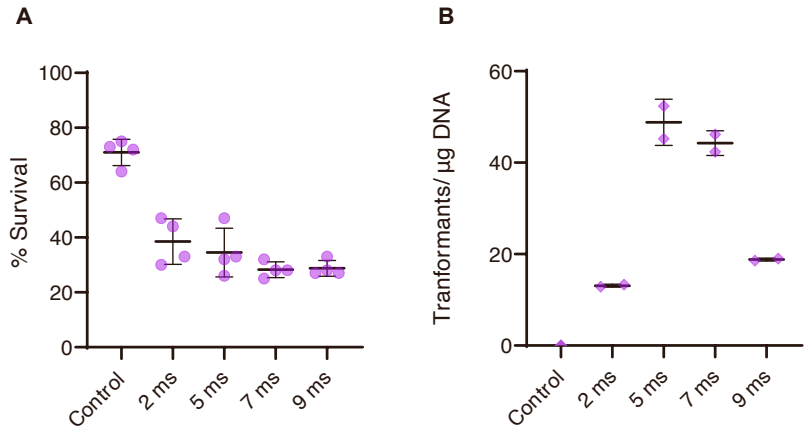

Figure S3. Transformation efficiency and survival rate of electroporation with different pulse duration. **Related to Figure 1**. (**A**) The survival rate of UM1 protoplasts after 2, 5, 7, and 9 milliseconds TC pulses. Each point corresponds to a different plate with 200 protoplasts (2 plates/cuvette). (**B**) Transformants/µg of DNA obtained with 2, 5, 7, and 9 milliseconds TC pulses. Each point corresponds to a different electroporation cuvette.

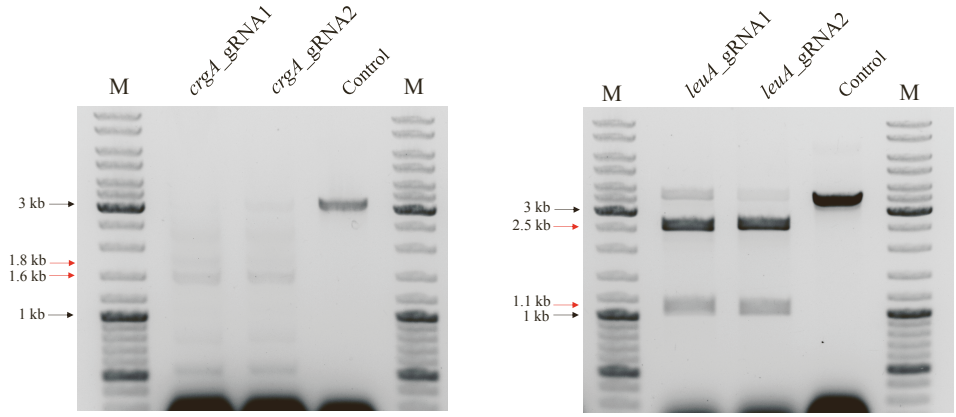

Figure S4. *In vitro* activity test of the crRNAs designed for *crgA* and *leuA* disruption. Related to STAR Methods. PCR amplified *crgA* (left) and *leuA* (right) loci were incubated with Cas9 and gRNA (tracRNA + crRNA) at 30 ºC for 2 hours. As a control, crRNA was removed to check unspecific cleaves within the targeted locus. Red arrows point to the expected fragments after the Cas9-gRNA cleave. Black arrows point to the indicated bands of the marker.

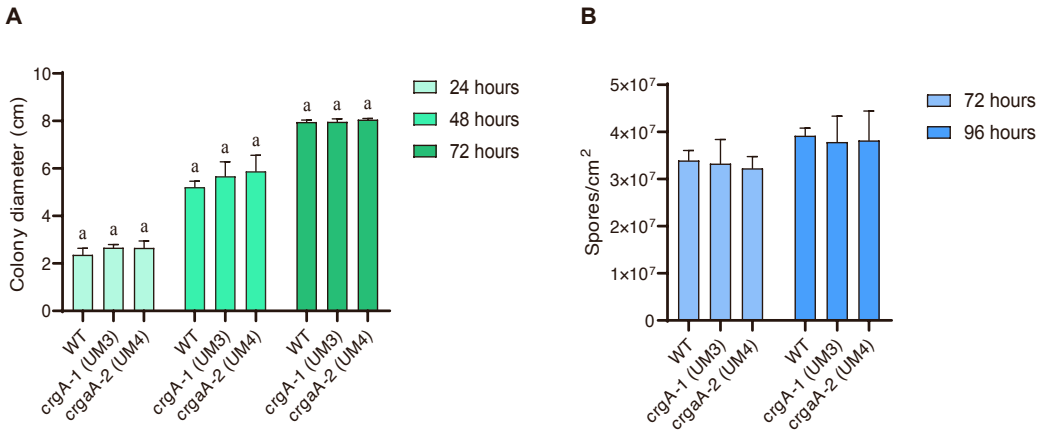

Figure S5. Radial growth analysis and spore production of the *cgrA* mutants UM3 and UM4 compared to the wild-type strain. **Related to Figure 4.** (**A**) Colony diameter of *R. microsporus* wild-type strain (WT) and *crgA* mutants UM3 and UM4 after 24, 48, 72 hours of growth in rich YPG media. (**B**) Total spore production by cm^2 quantified after 72 and 96 hours. The data were analyzed using one-way ANOVA, and statistical significance is indicated by letters ($p < 0,05$).

Table S1: crRNAs and primers name and sequence used in this work. Related to STAR Methods

Table 1.	
crRNA name	**Sequence 5'→ 3'**
*crgA*_1 (*crgA*_gRNA1)	GCAAGTGATCCGACCAATAG AGG
*crgA*_2 (*crgA*_gRNA2)	GCCCGATGGTCGATCGATCG TGG
*leuA*_1 (*leuA*_gRNA1)	CCTGGCACTACCGTCGTCTG CGG
*leuA*_2 (*leuA*_gRNA2)	CAAGGTATTGTGCACGTCAT TGG
Primer name	**Sequence 5'→ 3'**
*pyrF*_F_SEQ	GGCAATCATGTCATCGGAACT
*pyrF*_R_SEQ	GCTCAAGGTTGAATAAAGAC
*pyrG*_F_SEQ	CCCGCATAAATCCTTTTGAA
*pyrG*_R_SEQ	ACGGACCATTGGATACATCA
*pyrF*_F_EcoRI	CAGGAATTCGTATCTTGAGCTTACAAACGACTTG
*pyrF*_R_XhoI	CAGCTCGAGTGATAAAACGAAGATGTGGCTGTC
Templ_F_*crgA*_1	GATCTTGACCTAGAGCATGATACTCGAGTACCTCTATTCCTCCATAA GAATTTGACAG
Templ_R_*crgA*_1	TCATGCAGTTGACTCCAGGGAATGCAAGTGATCCGACCTGATAAAA CGAAGATGTGGCTGTC
Templ_F_*crgA*_2	TTGATGCAAGTACAAACCTTGCCCGATGGTCGATCGATTCCTCCATA AGAATTTGACAG
Templ_R_*crgA*_2	TGGTTACACGAAAGCGATGCGAGCCTACTGCTTCCACGTGATAAAA CGAAGATGTGGCTGTC
Templ_F_*leuA*_1	TGAACAAGGATTTACCCTTCCTGGCACTACCGTCGTCTTCCTCCATA AGAATTTGACAG
Templ_R_*leuA*_1	GCCAAATGCACCATGTGTTGAGGTGTGTGAGTCGCCGC TGATAAAACGAAGATGTGGCTGTC
Templ_F_*leuA*_2	TGGTATGGAAGATGCTCGTCAAGGTATTGTGCACGTCATCCTCCAT AAGAATTTGACAG
Templ_R_*leuA*_2	GGTAGTGCCAGGAAGGGTAAATCCTTGTTCAGGACCAATGATAAA ACGAAGATGTGGCTGTC
Up_F_*crgA*	ATGGCAATAACCAGACCATACC
Dw_R_*crgA*	AACATCCTTCTAGAACCGCGTA
Up_F_*leuA*	GCAGCAAAATCCACATAGTCAA
Dw_R_*leuA*	CGAAAAAGGAAATAACGCTTTG
*pyrF*_RC	TAGTCATGCGTCCAGTTTCTGT
M13_F	GTAAAACGACGGCCAGT
M13_R	CAGGAAACAGCTATGAC