Development of a CRISPR/Cas9-Based Tool for Gene Deletion in *Issatchenkia orientalis*

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**ABSTRACT** The nonconventional yeast *Issatchenkia orientalis* has emerged as a potential platform microorganism for production of organic acids due to its ability to grow robustly under highly acidic conditions. However, lack of efficient genetic tools remains a major bottleneck in metabolic engineering of this organism. Here we report that the autonomously replicating sequence (ARS) from *Saccharomyces cerevisiae* (ScARS) was functional for plasmid replication in *I. orientalis*, and the resulting episomal plasmid enabled efficient genome editing by the CRISPR/Cas9 system. The optimized CRISPR/Cas9-based system employed a fusion RPR1-tRNA promoter for single guide RNA (sgRNA) expression and could attain greater than 97% gene disruption efficiency for various gene targets. Additionally, we demonstrated multiplexed gene deletion with disruption efficiencies of 90% and 47% for double gene and triple gene knockouts, respectively. This genome editing tool can be used for rapid strain development and metabolic engineering of this organism for production of biofuels and chemicals.

**IMPORTANCE** Microbial production of fuels and chemicals from renewable and readily available biomass is a sustainable and economically attractive alternative to petroleum-based production. Because of its unusual tolerance to highly acidic conditions, *I. orientalis* is a promising potential candidate for the manufacture of valued organic acids. Nevertheless, reliable and efficient genetic engineering tools in *I. orientalis* are limited. The results outlined in this paper describe a stable episomal ARS-containing plasmid and the first CRISPR/Cas9-based system for gene disruptions in *I. orientalis*, paving the way for applying genome engineering and metabolic engineering strategies and tools in this microorganism for production of fuels and chemicals.

**KEYWORDS** CRISPR/Cas9, *Issatchenkia orientalis*, genome editing, metabolic engineering, synthetic biology

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Owing to its extraordinary tolerance to multiple stresses, including extremely low-pH conditions, *Issatchenkia orientalis* is a promising platform microorganism for the production of organic acids. It was previously used in ethanol fermentation at pH 2 (1) and engineered to produce succinic acid (2) and lactic acid (3). However, the tools for genetic engineering in *I. orientalis* are very limited, which significantly prohibits extensive metabolic engineering efforts. Stable episomal plasmids and efficient genome editing tools are the two foundational technologies for genetic engineering (4), which *I. orientalis* is currently lacking. Episomal plasmids allow rapid genetic manipulations and render microorganisms genetically tractable. The core functional element of episomal plasmids is an autonomously replicating sequence (ARS). ARS is a DNA replication starting point, similar to the origin of replication in bacteria, and it
directs the replication of the genomic DNA and episomal plasmid (5, 6). For *Saccharomyces cerevisiae*, episomal plasmids include centromere (CEN)-based low-copy-number plasmids and 2μ-based high-copy-number plasmids (4). In contrast, there is no available stable episomal plasmid for *I. orientalis*, and even a functional ARS has not been identified.

In addition to the lack of episomal plasmids, *I. orientalis* is devoid of precise genome editing tools, in particular CRISPR/Cas (clustered regularly interspaced short palindromic repeats and CRISPR-associated proteins). CRISPR/Cas9 is a powerful tool for rapid genome engineering in which a single guide RNA (sgRNA) containing a spacer sequence complementary to the targeted DNA sequence guides Cas9, a DNA endonuclease enzyme, to a genomic target (7, 8). Upon binding, Cas9 creates a DNA double-strand break (DSB). DNA repair mechanisms, homologous recombination (HR) or nonhomologous end joining (NHEJ), can be exploited to introduce gene insertions and deletions. CRISPR/Cas9 has been successfully implemented with high editing efficiencies in various species, such as *Escherichia coli*, *S. cerevisiae*, and mammalian cells (9–12). A CRISPR/Cas9-based tool for *I. orientalis*, however, has not yet been developed.

In this study, we report a functional ARS for plasmid replication and the first CRISPR/Cas9-based system for targeted and markerless gene disruption in *I. orientalis*. A variety of promoters for sgRNA expression were characterized, and knockouts of several genes were performed with efficiencies greater than 97%. We also demonstrated efficient multiplexed genome editing by disrupting *ADE2* and *TRP1*; *ADE2* and *HIS3*; and *ADE2*, *HIS3*, and *SDH2* with 72.8%, 89.9%, and 46.7% disruption efficiencies, respectively. Our optimized CRISPR/Cas9 system represents a powerful tool for comprehensive metabolic engineering of *I. orientalis* to produce biofuels and chemicals.

**RESULTS**

**Replicable plasmid endowed by *S. cerevisiae* ARS.** Based on a recent study on *I. orientalis* population genomics, there was a high similarity of the genome sequences between *I. orientalis* and *S. cerevisiae* (13). Hence, we hypothesized that the well-characterized ARS from *S. cerevisiae* (ScARS) may be functional in *I. orientalis*. To test this hypothesis, we used the DNA assembler method (14) to construct a plasmid (pIo-UG), which was derived from pRS415, containing the *I. orientalis* uracil auxotrophic selection marker (*IoURA3*), ScARS, *S. cerevisiae* LEU2 (ScLEU2), and a green fluorescent protein (GFP) gene as a reporter (Fig. 1A). Approximately 1000 colonies were obtained with 500 ng pIo-UG by heat shock transformation (see Fig. S1A in the supplemental material), and around 55% of the cells cultured in liquid media could express the GFP at a symmetric peak for at least 5 days (Fig. 1B). However, we did not observe any colony growing for the control plasmid pIo-control (without ScARS) on an SC-URA (SC-uracil) plate (Fig. S1B). Then, plasmid was extracted from *I. orientalis* cultured for 120 h and transformed to *E. coli*. We were still able to see *E. coli* colonies (Fig. S1C), and plasmid extracted from *E. coli* was confirmed to be the original plasmid pIo-UG by heat shock transformation (see Fig. S1A in the supplemental material), and around 55% of the cells cultured in liquid media could express the GFP at a symmetric peak for at least 5 days (Fig. 1B). However, we did not observe any colony growing for the control plasmid pIo-control (without ScARS) on an SC-URA (SC-uracil) plate (Fig. S1B). Then, plasmid was extracted from *I. orientalis* cultured for 120 h and transformed to *E. coli*. We were still able to see *E. coli* colonies (Fig. S1C), and plasmid extracted from *E. coli* was confirmed to be the original plasmid pIo-UG by restriction digestion (Fig. S1D). Thus, the ScARS-containing plasmid could be maintained in *I. orientalis*. It was also reported that CEN sequence could greatly improve plasmid stability in *S. cerevisiae*, and the plasmid with CEN showed >80% GFP expression (4). We tested the functionality of the centromere from *S. cerevisiae* (ScCEN) in *I. orientalis*. However, no improvement was obtained by the addition of ScCEN to pIo-UG (Fig. S1E). Since CEN is essential to direct precise DNA segregation, isolation of a functional CEN is the goal of our future work.

**CRISPR/Cas9 system in *I. orientalis*.** Having obtained a functional ARS, we next sought to design a CRISPR/Cas9-based tool for *I. orientalis*. An efficient CRISPR/Cas9 system requires functional Cas9 and sgRNA expressions. Cas9 expression can be achieved by using a constitutive RNA polymerase (RNAP) II promoter. On the other hand, sgRNA expression typically requires an RNAP III promoter because of the mRNA processing associated with RNAP II, such as 5’ end capping and 3’ end polyadenylation (15). Should RNAP II promoter be used for sgRNA expression, the sgRNA needs to be flanked with self-cleaving delta virus ribozyme sequences (16). These ribozymes can
execute cleavage on both ends of sgRNA and release the mature sgRNA without posttranscriptional modifications.

In yeasts, genes transcribed by the RNAP III promoter include all the tRNA genes, SNR6 (U6 spliceosomal RNA), SNR52 (C/D box small nucleolar RNA), RPR1 (RNA component of nuclear RNase P), SCR1 (RNA subunit of the signal recognition particle), and 5S rRNA (17). These promoters have been used to drive sgRNA expression in other yeast species. For example, SNR52 and 5S rRNA were used for sgRNA expression in S. cerevisiae and Aspergillus niger, respectively (10, 18). tRNA by itself can act as a promoter, and fusion of tRNA with other promoters, such as the hybrid promoter SCR1\textendash}tRNA\textsubscript{Gly} in Yarrowia lipolytica, can excise the sgRNA from the primary transcript by the tRNA maturation processing (19). Hence, it is possible to use these RNAP III promoters to express sgRNA in I. orientalis. The partial sequence of RPR1 in I. orientalis ATCC 6258 was previously identified (20), and there was a homolog in I. orientalis SD108. 5S rRNA in I. orientalis was identified by BLAST search against the 5S rRNA of S. cerevisiae S288C. These two genes serve as the starting point for sgRNA expression in our CRISPR/Cas9-based system.

A series of promoters was evaluated, including a leucine tRNA (tRNA\textsubscript{Leu}), a serine tRNA (tRNA\textsubscript{Ser}), 5S rRNA, RPR1, and fusions of 5S rRNA and RPR1’ with tRNA\textsubscript{Leu} (Fig. 2A). The RPR1 promoter contains 250 bp upstream of RPR1 partial sequence, while the RPR1’ promoter contains 250 bp upstream of RPR1 and first 120 bp of RPR1. The promoter elements of RPR1 can be located upstream or internal to the mature product (17). However, the exact promoter elements of RPR1 from I. orientalis are unknown. Therefore, we tested two different RPR1 promoters. The Cas9 used in this system is iCas9, which is short for improved Cas9 and was shown to yield higher disruption efficiency in S. cerevisiae than the wild-type Cas9 (21). iCas9 was tagged with simian virus 40
nuclear localization sequences at both N and C termini and driven by a strong constitutive promoter, TEF1ap.

As a proof of concept, we targeted the ADE2 gene because the ADE2 nonsense mutant shows a conspicuous red phenotype, which could be screened for quickly (21). Initially, we did not know whether HR or NHEJ was the dominant repairing mechanism in *I. orientalis*. First, we evaluated the HR mechanism by including an HR disruption in the plasmid. A gBlock containing an HR donor and spacer for ADE2 disruption was cloned into CRISPR/Cas9 plasmid using the Golden Gate assembly method (22), and in the resulting plasmid, the HR donor was fused to the 5’ end of sgRNA (Fig. 2B). The HR donor contained an 8-bp deletion in the middle, and two 50-bp-homology arms flanked both sides of the centered 8-bp deletion (21). The 8-bp deletion included the protospacer adjacent motif (PAM) sequence and the last 3 bp of the spacer. If HR was the primary mode of DNA DSB repair, the defined 8 bp would be removed from the genome, resulting in a frameshift mutation. Various CRISPR/Cas9 constructs with different promoters for sgRNA expression were transformed into *I. orientalis*, and all transformants were plated on SC-URA plates. Colonies were then screened for the red and white phenotypes. For all cases, growing the cells for a prolonged period of time in liquid SC-URA after transformation was not necessary to observe ADE2 disruption.

The highest ADE2 disruption efficiency of 97.0% ± 1.0% (number of red colonies/number of total colonies: 200/209, 320/326, 300/309) was attained with RPR1-tRNALeu.
The RPR1 and 5S RNA-tRNALeu promoters also produced high-efficiency ADE2 disruptions, 93.3% ± 0.9% (140/150, 128/139, 100/106) and 89.8% ± 1.2% (200/227, 190/210, 200/220), respectively. The tRNALeu, tRNASer, and 5S rRNA promoters resulted in lower disruption efficiencies, 84.4% ± 1.9% (250/306, 280/327, 320/373), 76.9% ± 1.3% (141/187, 115/150, 110/140), and 67.8% ± 1.6% (4/6, 7/10, 4/6), respectively. To confirm ADE2 disruption, 4 red colonies were randomly picked and sent for sequencing, and DNA sequencing analysis showed deletion of the defined 8 bp for all colonies (Fig. S2A), indicating that our CRISPR/Cas9 system was functional for gene disruption and *I. orientalis* could utilize HR to repair DNA DSB.

Next, to test if *I. orientalis* was capable of accomplishing NHEJ-based DNA repair, only the spacer was cloned into the CRISPR/Cas9 plasmid with RPR1-tRNALeu promoter. Following transformation of *I. orientalis* with the plasmid, less than 10 transformants appeared, and they all retained the wild-type white color (Fig. S2B). In addition, plasmid with RPR1-tRNALeu promoter lacking both HR donor and sgRNA was transformed into *I. orientalis*,

**FIG 3** ADE2 disruption. (A) ADE2 knockout efficiencies using different promoters for sgRNA expression. All asterisks indicate statistical difference (*P* < 0.05) calculated using a two-tailed type II Student t test. Error represents standard deviation for biological triplicates. (B) qPCR analysis of sgRNA expression levels for different promoters. Data shown are sgRNA level normalized to the sgRNA level generated by the tRNA<sup>Leu</sup> promoter. alg9 was used as the reference gene. Error bars represent standard deviations for biological triplicates.
and the number of colonies appearing was greater than $10^5$ (Fig. S2C). Taken together, these data suggested that HR is the main DNA repair mechanism.

To determine whether sgRNA expression levels correlate with ADE2 disruption efficiencies, qPCR was employed to quantify the transcription levels of sgRNAs (Fig. 3B). Transcript levels produced from tRNA$^{Leu}$, tRNA$^{Ser}$, and 5S rRNA promoters were lower than those produced from other promoters, which might explain the lower ADE2 knockout efficiencies. RPR1 promoter produced approximately 2-fold more sgRNA than the RPR1$^-$tRNA$^{Leu}$ promoter, yet the ADE2 disruption efficiency by the RPR1 promoter was not as high as that by the RPR1$^-$tRNA$^{Leu}$ promoter.

To validate if the RPR1$^-$tRNA$^{Leu}$ promoter would consistently produce higher knockout efficiency, despite the RPR1 promoter producing the largest amount of sgRNA, several additional genes were chosen for disruptions using both RPR1 and RPR1$^-$tRNA$^{Leu}$ promoters to express sgRNA. The disruption efficiencies between these promoters were then compared. LEU2, HIS3, and TRP1 are essential for yeasts to synthesize their own leucine, histidine, and tryptophan, respectively. Successful disruptions of these genes will also result in mutants with leu2, his3, or trp1 auxotrophy. Random colonies were picked and screened for knockout using liquids SC-URA and SC minus appropriate auxotrophic compound. A disruption efficiency of 100% (10/10, 10/10) was obtained for LEU2 and TRP1 knockouts for both RPR1 and RPR1$^-$tRNA$^{Leu}$ promoters (Table 1 and Fig. S3). However, the efficiency of HIS3 disruption by RPR1 promoter, 90% ± 10% (8/10, 10/10), was lower than that by RPR1$^-$tRNA$^{Leu}$ promoter, 100% (10/10, 10/10). Five colonies from each disruption using RPR1$^-$tRNA$^{Leu}$ promoter were sent for sequencing, and they were all confirmed to be disrupted at the defined target site. Since RPR1$^-$tRNA$^{Leu}$ promoter generally resulted in higher disruption efficiencies, it was chosen as the final promoter for sgRNA expression for subsequent knockouts.

To also demonstrate that our CRISPR/Cas9 system can be used to knock out genes whose disruptions do not result in visible phenotypic change, SDH1 and SDH2, encoding subunits 1 and 2 of succinate dehydrogenase, respectively, were chosen for single-gene and triple-gene disruption. In the SDH1 knockout, 8 colonies were randomly picked and screened by PCR and DNA sequencing, and 7 out of 8 colonies were shown to be disrupted at the chosen target site (Fig. 4A).

### Table 1: Comparison of disruption efficiencies between RPR1 and RPR1$^-$tRNA$^{Leu}$ promoters

| Gene   | Disruption efficiency by promoter (%) |
|--------|---------------------------------------|
|        | RPR1 | RPR1$^-$tRNA$^{Leu}$ |
| LEU2   | 100  | 100                |
| HIS3   | 90 ± 10 | 100              |
| TRP1   | 100  | 100                |

Multiple gene disruption using CRISPR/Cas9. In addition to single-gene knockouts, we examined our CRISPR/Cas9 system for multiplexed gene deletions. Cassettes for sgRNA expressions were separately PCR amplified and sequentially cloned into CRISPR/Cas9 plasmid (Fig. 5). ADE2 and TRP1 and ADE2 and HIS3 were selected for double-gene knockouts. The multiplexed CRISPR plasmid was transformed into I. orientalis, and all transformants were plated on an SC-URA plate. Independently of the TRP1 or HIS3 knockout efficiency, the overall ADE2 editing efficiencies were determined to be $89.5\% \pm 0.6\%$ (113/127, 100/111) and $95.9\% \pm 0.7\%$ (200/210, 200/207) for ADE2/TRP1 and ADE2/HIS3 double-gene knockouts, respectively. For each knockout, 8 randomly picked red colonies were then screened for loss of TRP1 or HIS3 function using SC-URA and SC lacking appropriate auxotrophic nutrients. TRP1 and HIS3 disruption efficiencies were determined to be $81.3\% \pm 6.3\%$ (6/8, 7/8) and $93.8\% \pm 6.3\%$ (8/8, 7/8), respectively (Fig. S4). All things considered, in double-gene knockouts, ADE2 and TRP1 and ADE2 and HIS3 were deleted with efficiencies estimated to be $72.8\% \pm 6.0\%$ and $89.9\% \pm 5.3\%$, respectively (Table 2).
Last, we also tested whether our CRISPR/Cas9 system could disrupt 3 genes simultaneously in a single transformation. ADE2, HIS3, and SDH2 were selected as target genes. The overall disruption efficiency of ADE2 was estimated to be 62.3% (114/183). Eight red colonies were then randomly picked and screened for HIS3 and SDH2 knockouts by sequencing. Among these 8 colonies, 6 of them had both HIS3 and SDH2 disrupted (Fig. 4B and C). Thus, ADE2, HIS3, and SDH2 were simultaneously disrupted with an efficiency of 46.7% (Table 2).

**DISCUSSION**

The yeast *I. orientalis* is renowned for its unique ability to tolerate several stresses, including extremely low pH (2). Thus, it is a microorganism that can potentially be engineered to produce organic acids. Nevertheless, engineering endeavors in this species will be hindered until efficient and consistent synthetic biology tools are established. In this study, we determined that the ScARS allowed stable plasmid replication and maintenance in *I. orientalis*. Around 55% of the cells grown for 5 days in liquid culture still carried the plasmid and expressed GFP. This episomal plasmid will be a useful tool for rapid genetics and allow expression of biological parts without permanently integrating the parts into the chromosome.

CRISPR/Cas9 is an emerging genome engineering tool that has revolutionized biotechnology research (7). Developing a CRISPR/Cas9 system in *I. orientalis* posed some technical challenges. RNAP III promoters in many nonconventional yeasts, including *I. orientalis*, are not well characterized (23). We were unable to identify the potential sequences for the endogenous SNR52 and SCR1. It might be possible that these promoters could achieve even greater gene disruption efficiency than our
Furthermore, *I. orientalis* is diploid, and diploid and polyploid industrial yeasts can be particularly challenging to engineer due to the difficulty and necessity of disrupting multiple copies of one gene (24). Despite these challenges, we have developed a CRISPR/Cas9-based tool that could achieve high gene disruption efficiency in *I. orientalis*. We examined several promoters for sgRNA expression and found that the RPR1 and RPR1'-tRNALeu promoters exhibited high ADE2 disruption rates. The RPR1'-tRNALeu promoter increased ADE2 knockout efficiency slightly but significantly compared to RPR1 promoter (*P = 0.0182*) (Fig. 2B). Furthermore, qPCR results indicated that the RPR1 promoter produced more sgRNA transcripts than the RPR1'-tRNALeu promoter, but efficiencies of gene knockout by the RPR1'-tRNALeu promoter were generally higher for various single-gene targets. Nevertheless, it is not unusual that the promoter with the highest sgRNA expression does not result in the highest disruption efficiency. It was reported that in *Y. lipolytica*, the SNR52'-tRNA Gly promoter produced the largest amount of sgRNA, around 2-fold more sgRNA than the SCR1'-tRNA Gly promoter (19). However, the highest PEX10 disruption efficiency was achieved by using the SCR1'-tRNA Gly promoter.

Our CRISPR/Cas9 system also could achieve high efficiency in multiplexed gene

**FIG 5** Scheme showing the assembly of CRISPR/Cas9 plasmid for multiplexed gene deletions. gBlock containing HR donor and spacer for each target site was first assembled into pVT36b. sgRNA expression cassettes were then PCR amplified, and the Golden Gate assembly method was used to clone the cassettes into pVT36z.

**TABLE 2** Multiplexed gene disruption efficiencies

| Genes            | Disruption efficiency (%) |
|------------------|---------------------------|
| ADE2 and TRP1    | 72.8 ± 6.0                |
| ADE2 and HIS3    | 89.9 ± 5.3                |
| ADE2, HIS3, and SDH2 | 46.7                     |
deletions, 90% and 47% for double-gene and triple-gene disruptions, respectively. In haploid _S. cerevisiae_, triple-gene disruption could be achieved with an efficiency of 100% (21). In the industrial triploid _S. cerevisiae_ strain Ethanol Red, four genes were disrupted in a single transformation with 100% efficiency as well (25). We demonstrated triple-gene knockout in diploid _I. orientalis_ was possible, although the disruption efficiency was not as high as in _S. cerevisiae_. In comparison to other nonconventional yeasts, double- and triple-gene knockouts were achieved in _Y. lipolytica_ with efficiencies of 36.7% and 19.3%, respectively (26). These efficiencies were lower than our reported ones. Furthermore, in a recently published paper, a CRISPR/Cas9-based system was developed in _Rhodosporidium toruloides_ and could achieve single- and double-gene knockouts with efficiencies of 95% and 78%, respectively (27), which are comparable to our single- and double-gene disruption efficiencies. Nevertheless, triple-gene knockout was not shown in _R. toruloides_.

One major concern of the CRISPR/Cas9 technology is the likelihood and unknown consequences of off-target effects (28). However, CRISPR off-target effects could be readily detected in _I. orientalis_. Efficient CRISPR-mediated genome editing in _I. orientalis_ relied on the Cas9-induced DSB at the target site and the rescue of DSB lethality by HR. Successful genomic integration of HR repair template then resulted in deletion of the PAM sequence and the last 3 bp of the protospacer, which prevented repeated cleavage by CRISPR/Cas9. If an sgRNA caused nonspecific DSB, DNA repair through HR was unlikely to occur because of the lack of HR disruption donor with homology to the off-target site. Moreover, Cas9 would cause continuous cleavage since the sgRNA target site was not removed. Thus, after transformation of _I. orientalis_ with CRISPR/Cas9 plasmid containing both sgRNA and HR disruption donor, if off-target activity happened, very few transformants would survive.

Taken together, we expanded the genetic engineering toolkit for _I. orientalis_ with the development of the episomal plasmid and the CRISPR/Cas9-based system. These tools have laid the foundation for CRISPR/Cas9-mediated genome editing in _I. orientalis_. They also serve as the steppingstone that will allow development of complex genome-scale engineering tools, such as CRISPR-AID (8), and further increase the engineering capacity in _I. orientalis_. Future efforts include identification of a CEN sequence and further optimization of our CRISPR/Cas9 system. While ARS-containing plasmid can replicate, it is mitotically unstable and lost at high frequency after each cell division (29). Addition of a CEN to an ARS-containing plasmid helps increase the stability of the plasmid and transmission of the plasmid to daughter cells (30). Other sgRNA expression methods, such as gRNA-tRNA array (31), could potentially increase the multiplexed gene deletion efficiency.

**MATERIALS AND METHODS**

**Strains, media, and chemicals.** The strains used in this study are listed in Table S1 in the supplemental material. _E. coli_ transformants were grown at 37°C in LB medium supplemented with 100 μg/ml ampicillin. _S. cerevisiae_ Y5GS0 and _I. orientalis_ SD108 and its mutants were propagated at 30°C in YPAD medium (1% yeast extract, 2% peptone, 0.01% adenine hemisulfate, and 2% dextrose). Yeast transformants were cultured or selected in the Synthetic Complete (SC) dropout medium lacking uracil, tryptophan, leucine, or histidine or with a low concentration of adenine (~10 mg/liter) (SC-URA, SC-TRP, SC-LEU, SC-HIS, or SC-ADE, respectively). DNA polymerase and restriction enzymes were purchased from New England Biolabs (Ipswich, MA). DNA extraction and purification kits were purchased from Zymo Research (Irvine, CA). All other chemicals were purchased from Sigma (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA). Oligonucleotides, including gBlocks and primers, were all synthesized by Integrated DNA Technologies (IDT; Coralville, IA).

**Plasmid construction.** The plasmid pLo-UG was constructed using the DNA assembler method developed previously (14). In brief, the PCR-amplified fragments, GFP cassette (with TDH3p and Tef1at) and IoURA3 (with URA3p and ENO2t), were cotransformed with Apal- and NotI-digested pRS415 backbone into _S. cerevisiae_ for _in vivo_ assembly via electroporation or lithium acetate-mediated methods. The isolated yeast plasmids were then transformed into _E. coli_ for enrichment, and their identities were verified by restriction digestion or DNA sequencing. The correctly assembled plasmids were subsequently transformed into _I. orientalis_ SD108 for target gene expression.

CRISPR/Cas9 plasmids were constructed using DNA assembler from gBlocks containing promoter for sgRNA expression and the following fragments PCR amplified from previous constructs: iCas9, _I. orientalis_ URA3 expression cassette, _E. coli_ helper fragment, and _S. cerevisiae_ URA3 expression cassette flanked by
XhoI recognition sites and CEN6/ARS4. The resulting plasmids were digested with XhoI to remove S. cerevisiae URA3 expression cassette and religated. The HR donor and spacer sequences were ordered as gBlocks and assembled into CRISPR/Cas9 plasmids by the Golden Gate assembly method (22). Plasmids, key primers, gBlocks, and sequences of RNAP III promoters can be found in the supplemental material (Tables S1 to S4).

Transformation of I. orientalis and its derived mutants. A fresh 2-ml overnight YPAD culture of I. orientalis was diluted to an initial optical density at 600 nm (OD$_{600}$) of 0.2. The cells were continuously grown until they reached an OD$_{600}$ of 0.8 to 1. Cells were collected by centrifugation, washed twice with deionized water, and resuspended in 360 μl of transformation mixture containing 240 μl of 50% (wt/vol) polyethylene glycol (PEG) 3350, 36 μl of 1 M lithium acetate, 50 μl of 2-mg/ml DNA from salmon testes (SS-DNA) that was boiled at 100°C for 5 min and quickly chilled on ice, plasmid (1 μg), and deionized water. After mixing thoroughly, the suspension was subjected to heat shock for 1 h at 42°C. Cells were collected by centrifugation and spread on appropriate plates.

Flow cytometry analysis. The GFP expression was measured by flow cytometry as described elsewhere (32). Briefly, the transformed I. orientalis cells were cultured in SC-URA medium for ~24 to 120 h and then centrifuged for 2 min at 2,000 × g to remove the supernatant. The cell pellets were resuspended in 10 mM phosphate-buffered saline (pH 7.4) and then analyzed by flow cytometry at 488 nm on a BD LSR II flow cytometer analyzer (BD Biosciences, San Jose, CA). After flow cytometry analysis, the I. orientalis plasmids were extracted with the Zymoprep Yeast Plasmid Miniprep II kit and retransformed to E. coli for colony counting.

qPCR. I. orientalis cultures were inoculated from a plate and grown in SC-URA medium until mid-log phase (OD 2 to 3). Total RNA was extracted using the Qiagen RNeasy kit (Venlo, Netherlands), and reverse transcription was performed with the Bio-Rad iScript cDNA synthesis kit (Hercules, CA), with a prior denaturation step at 65°C for 5 min to disrupt gRNA secondary structure. qPCR was performed using Bio-Rad iTaq Universal SYBR green Supermix on a Roche LightCycler 480 qPCR system. alg9 was used as the reference gene for relative quantification. Experiments were done in biological triplicate.

Genomic DNA extraction and target site sequencing. Genomic DNA extraction of I. orientalis was performed using methods described elsewhere (33). In brief, 200 μl cell culture was spun down and suspended in 200 μl lithium acetate (200 mM) with 1% SDS. The mixture was incubated at 70°C for 30 min. Then, 600 μl 100% ethanol was added to the mixture. After vortexing, DNA and cell debris were collected by centrifugation and washed with 70% ethanol. The pellet was dissolved in 50 μl water. After mixing thoroughly, the suspension was subjected to heat shock for 1 h at 42°C. Cells were collected by centrifugation and spread on appropriate plates.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mSphere.00345-19.

TEXT S1, DOCX file, 0.01 MB.

FIG S1, TIF file, 2.3 MB.

FIG S2, TIF file, 2.5 MB.

FIG S3, TIF file, 2.6 MB.

FIG S4, TIF file, 2.2 MB.

TABLE S1, DOCX file, 0.01 MB.

TABLE S2, DOCX file, 0.01 MB.

TABLE S3, DOCX file, 0.01 MB.

TABLE S4, DOCX file, 0.01 MB.

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