Development of a CRISPR/Cas9 system for high efficiency multiplexed gene deletion in *Rhodosporidium toruloides*

J. Carl Schultz | Mingfeng Cao | Huimin Zhao

1Department of Chemical and Biomolecular Engineering, Carl R. Woese Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, Illinois
2Departments of Chemistry, Biochemistry, and Bioengineering, University of Illinois at Urbana-Champaign, Urbana, Illinois

Correspondence
Huimin Zhao, Department of Chemical and Biomolecular Engineering, Carl R. Woese Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801.
Email: zhao5@illinois.edu

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Abstract
The oleaginous yeast *Rhodosporidium toruloides* is considered a promising candidate for production of chemicals and biofuels thanks to its ability to grow on lignocellulosic biomass, and its high production of lipids and carotenoids. However, efforts to engineer this organism are hindered by a lack of suitable genetic tools. Here we report the development of a CRISPR/Cas9 system for genome editing in *R. toruloides* based on a fusion 5S rRNA-tRNA promoter for guide RNA (gRNA) expression, capable of greater than 95% gene knockout for various genetic targets. Additionally, multiplexed double-gene knockout mutants were obtained using this method with an efficiency of 78%. This tool can be used to accelerate future metabolic engineering work in this yeast.

Keywords
CRISPR/Cas9, genome editing, metabolic engineering, multiplex, *Rhodosporidium toruloides*

1 INTRODUCTION

The oleaginous yeast *Rhodosporidium toruloides* is viewed as a potential platform organism for the production of both fatty acid- and terpenoid-derived compounds (Yaegashi et al., 2017; Zhang, Skerker et al., 2016). Combined with its ability to grow on lignocellulosic sugars such as xylose and arabinose (Zhang, Ito, Skerker, Arkin, & Rao, 2016), these capabilities make this yeast a promising candidate for production of value-added compounds from biomass. To date, efforts have been made to engineer the yeast for improved production of lipids and carotenoids (Pi et al., 2018; Zhang, Skerker et al., 2016) as well as nonendogenously produced compounds such as fatty alcohols or terpenoids through introduction of heterologous genes (Fillet et al., 2015; Yaegashi et al., 2017). However, these efforts are hampered by the limited availability of genetic engineering tools in this species, and the fact that nonhomologous end joining (NHEJ) dominates over homologous recombination (HR) for DNA repair. Gene knockout is an essential tool for performing metabolic engineering. In *R. toruloides*, the only demonstrated method of targeted gene knockout relies on the deletion of the NHEJ-related gene KU70, causing the cells to switch to predominantly HR-based DNA repair. However, this has been observed to decrease the cells' overall DNA repair capability as well as the transformation efficiency (Koh, Liu, Moehninsi, Du, & Ji, 2014). For metabolic engineering purposes, gene knockout methods that do not compromise the cells' robustness are preferable. Additionally, homology-based genetic engineering approaches require the tedious cloning of homology arms, which is made more challenging in this organism by its high GC content.

The CRISPR/Cas9 system, which has revolutionized the genetic engineering process for many organisms (Hsu, Lander, & Zhang, 2014), offers a solution to these challenges. By changing only a 20 nucleotide guide RNA (gRNA) sequence, the Cas9 enzyme can introduce precise cuts to most loci in the genome, allowing for targeted and markerless genetic manipulations (C. Schwartz, Shabbir-Hussain, Frogue, Blenner, & Wheeldon, 2017). In this study, we report the first development of a functional CRISPR/Cas9 system in *R. toruloides* for modular, targeted gene knockout. Different Cas9 and gRNA expression systems were evaluated, and reporter genes in the β-carotene biosynthetic pathway (phytoene synthase, phytoene desaturase) as well as the auxotrophic selection marker LEU2 (β-isopropylmalate dehydrogenase) were knocked out to assess the deletion efficiency amongst different targets. Finally, we
demonstrate the capability of this method to perform multiplex gene deletion in *R. toruloides*.

For the CRISPR/Cas9 system to function, a Cas protein and RNA guide (either a single gRNA, or a crRNA and tracrRNA) must be coexpressed in the nucleus of the cell. While a number of constitutive and inducible RNA Polymerase (RNAP) II promoters, suitable for expression of proteins, have been reported (Johns, Love, & Aves, 2016; Wang et al., 2016), there are no reported RNAP III promoters in *R. toruloides* for expression of short RNA molecules with well-defined ends, such as a gRNA. The commonly used RNAP III SNR52 promoter from *Saccharomyces cerevisiae* does not have a known ortholog in *R. toruloides*. As gRNA expression has proven to be a similar challenge in other nonmodel yeasts and fungi (C. M. Schwartz, Hussain, Blenner, & Wheeldon, 2016; Weninger, Hatzl, Schmid, Vogl, & Glieder, 2016; Zheng et al., 2018), we first sought to characterize several of the approaches that have been used previously in other species, in *R. toruloides*.

RNAP II promoters are typically not suitable for gRNA expression due to the G-cap and polyA tail which are added to the RNA transcript, but are not desirable for the CRISPR/Cas9 system (Weninger et al., 2016). However, the ribozyme-guide-ribozyme (RGR) system developed by Gao and Zhao (2014) fused the gRNA with hammerhead (HH) and hepatitis delta virus (HDV) ribozyme sequences which self-cleave at their 3' and 5' ends, respectively, allowing a HH-gRNA-HDV cassette to be expressed by a RNAP II promoter, whereupon the ribozymes will self-cleave and release the mature gRNA.

Furthermore, while the SNR52 snoRNA has not been reported in *R. toruloides*, other short RNAs such as transfer ribonucleic acid (tRNAs) and the 5S rRNA transcribed by RNAP III have been shown to have sufficient internal promoter elements to be used for expression of CRISPR gRNAs, by placing them immediately 5' of the gRNA sequence (C. M. Schwartz et al., 2016; Zheng et al., 2018). Therefore, we also investigated the use of a tRNA gene and the 5S rRNA as gRNA promoters in our initial designs. Notably, tRNA molecules will self-cleave from the gRNA transcript as they mature the following transcription, while the 5S rRNA does not.

Expression cassettes were designed for RGR, tRNA\textsuperscript{Gly}, and 5S-based gRNA expression (Figure 1a and Table S1) and assembled into the integration region of an ATMT plasmid (pEGFP-Rt-YR-HYG [Johns et al., 2016], replacing eGFP for the RGR cassette, inserting into p2PK-PGPD\_HYG-Tnos [Wang et al., 2016] for the others) and integrated to *R. toruloides* strain NP11 with the hygromycin (Hyg) selection marker.

Relative gRNA expression levels were quantified using quantitative polymerase reaction (qPCR; Figure 1b). While all strategies showed a higher expression for the gRNA sequence than the negative control (wild-type NP11), the 5S rRNA gave drastically higher expression than the tRNA, which was in turn much higher than the RGR method. These results are both in accordance with prior studies comparing the RGR approach to tRNA, and the tRNA approach to the 5S rRNA (C. M. Schwartz et al., 2016; Zheng et al., 2018).

For expression of Cas9, as it has been previously reported (Lin et al., 2014) that the high-GC codon bias of *R. toruloides* necessitates codon optimization for effective heterologous gene expression, a codon-optimized SpCas9 gene was ordered from GenScript (Piscataway, NJ). It has also been previously reported that the commonly used SV40 NLS is insufficient for nuclear targeting when located only at the protein C-terminus in *R. toruloides*. However, an endogenous NLS ("NLS3"), identified from the C-terminus of a putative transcription factor proved sufficient to enable recombinase-mediated selection marker recovery (Sun et al., 2018). Therefore, NLS3 was appended to the C-terminus of the codon-optimized SpCas9, and the construct was cloned into the ATMT plasmid pEFGP-Rt-YR-G418, replacing eGFP, to create pNM9-SpCas9-NLS3. The sequence of SpCas9-NLS3 is provided in Table S1.

Various genes in the \( \beta \)-carotene pathway (CRTYB, phytoene synthase/lycopene cyclase, CRTI, phytoene desaturase) have previously been used as reporter genes for targeted gene knockout in *R. toruloides* as their removal results in an albino phenotype of the otherwise red yeast (Koh et al., 2014; Zhang et al., 2016). For our

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**FIGURE 1** (a) Promoter designs used for gRNA expression in *Rhodosporidium toruloides*. Triangles indicate tRNA or ribozyme-mediated cleavage sites. (b) Quantitative polymerase chain reaction results for gRNA expression methods utilized. Data are normalized to the baseline \( C_\text{T} \) measured in the wild-type. Phosphoglycerate kinase was used as the reference gene. Error bars represent standard deviation of biological duplicates. gRNA, guide RNA; tRNA, transfer ribonucleic acid [Color figure can be viewed at wileyonlinelibrary.com]
system, SpCas9 under the strong constitutive phosphoglycerate kinase (PGK1) promoter was integrated to R. toruloides NP11, and a single clone was chosen to subsequently integrate a gRNA expression cassette targeting the CRTYB reporter gene expressed by each of our three strategies outlined above. Use of a single clone ensures Cas9 is integrated to the same genomic locus while comparing each gRNA expression method. While strains expressing the gRNA by the RGR and tRNA strategies did not show any knockouts, the strain expressing the gRNA using the 5S rRNA as a promoter showed a small (<1%) number of white colonies (Figure 2a). Sequencing of the genomic DNA confirmed that the gene knockouts were effected by CRISPR/Cas9 as deletions were visible at the protospacer adjacent motif (PAM) site of the gRNA (Figure 2c).

For effective genetic engineering, gene editing rates should be as close to 100% as possible. To improve the CRISPR efficiency to levels more useful for genetic engineering work, a number of further strategies were used. Previous work has shown that in some cases, presence of an N- and C-terminal NLS can be necessary for Cas9 activity (Lian, Hamedirad, Hu, & Zhao, 2017). Therefore, we cloned the SV40 NLS, NLS3, and four additional putative endogenous NLS sequences onto the N-terminus of SpCas9 (Table S2) and integrated the NLS-Cas9-NLS3 constructs to the genome of an NP11 single clone already containing a CRTYB-targeting gRNA expression cassette under the 5S rRNA promoter. However, none of these additional N-terminal NLS sequences noticeably improved Cas9 activity above that observed for Cas9-NLS3 (Table S3).

To observe the effect of modulating the Cas9 expression level, the PGK1 promoter was replaced by several other reported constitutive promoters, pGPD1, pFBA1, pPG11, and a putative TEF1 promoter. Each promoter-Cas9 construct was integrated to the genome of an NP11 single clone already containing a CRTYB-targeting gRNA expression cassette under the 5S rRNA promoter. While most promoters showed no improvement relative to pPGK1, in the case of pGPD1-driven expression, the gene knockout efficiency was observed to increase to 10% (Table S4). While pGPD1 is reported to be ~3-fold weaker than pPGK1 in R. toruloides (Wang et al., 2016), it has been previously reported in Pichia pastoris that higher Cas9 expression levels did not necessarily correlate with better editing efficiency (Weninger et al., 2016). The reason was hypothesized to be due to toxicity caused by excessively high Cas9 expression levels.

We also attempted to improve on the design of the 5S rRNA expression system. It was suggested that while sometimes having a long 5′ leading sequence on the gRNA may allow CRISPR to function, for certain guides (such as those with complementarity to the leading sequence) the efficiency will be significantly lowered (Weninger et al., 2016). Therefore, we attempted to place a tRNAArg gene between the 5S rRNA and the gRNA, reasoning that the stronger 5S promoter will produce high levels of gRNA transcript, while the tRNA will mature and release the gRNA without any extraneous 5′ sequence, allowing for more effective CRISPR activity from the transcripts produced. A similar approach of fusing a RNAP III promoter element with a tRNA gene for gRNA expression enabled highly efficient gene editing in Yarrowia lipolytica (C. M. Schwartz et al., 2016). In an additional design, drawing inspiration from the LEGO method developed by Deaner and coworkers, we placed a 5S-tRNA^Gly^-gRNA-tRNA^Arg^ cassette under the expression of the RNAP II PGK1 promoter in an attempt to further increase gRNA transcript levels (Deaner, Holzman, & Alper, 2018; Figure 1a and Table S1). qPCR indicates that both modifications resulted in lower levels of gRNA expression compared to the original 5S promoter (Figure 1b), but that the 5′ leading sequence was indeed cleaved successfully greater than 99% of the time in both cases (Figure S1). In this system, we found the additional RNAP II promoter-terminator set lowered the level of gRNA transcript. In the original report of the LEGO method, it was found that the effect of these additional elements varied depending on the tRNA genes being used.

These modified RNA expression systems were transformed into three individual NP11 clones each expressing SpCas9-NLS3 under either the PGK1 or GPD1 promoter. The stronger and weaker Cas9 promoters were both tested based on the finding in P. pastoris that combinatorial optimization of Cas9 and gRNA expression can be

**FIGURE 2** CRTYB knockout using pPGK1-SpCas9-NLS 3. (a) gRNA expression driven by 5S rRNA. (b) gRNA expression driven by 5S-tRNA fusion promoter. (c) Sequence alignment of CRTYB target site from albino colonies. gRNA is shown in bold and PAM sequence is underlined. gRNA, guide RNA; tRNA, transfer ribonucleic acid [Color figure can be viewed at wileyonlinelibrary.com]
necessary to achieve the highest editing efficiency (Weninger et al., 2016). Remarkably, greater than 99% gene knockout was observed using the 55-tRNA promoter with either Cas9 promoter (Figure 2b), while the additional flanking RNAP II promoter-terminator produced a somewhat lower knockout rate of 81 ± 10% with pGPD1-Cas9 and 84 ± 8% with pPGK1-Cas9, likely in accordance with its lower gRNA transcript level. As little difference was observed between pPGK1 and pGPD1 for Cas9 expression under the 55-tRNA promoter system, pPGK1 was used for Cas9 expression and the 55-tRNA promoter for gRNA expression for all subsequent work.

To verify the generality of this CRISPR/Cas9 system, additional genomic loci were targeted. In total, two guides each were used to attempt knockout of CRY1, CRTI, and LEU2. Knockout efficiencies in the carotene pathway were consistently at 95% efficiency or higher, while the LEU2 knockout rates were more moderate (Table 1). We hypothesize this is due to selection bias against the successful knockouts of this metabolically important gene. A similar effect has been reported in auxotrophs of LEU2 in S. cerevisiae, which was thought to be due to rate-limiting uptake of the auxotrophically required compound (Prong, 2002). Sequences of the gRNAs used are provided in Table S5.

As double-strand breaks are known to cause cell death, the degree of toxicity caused by CRISPR/Cas9 editing in R. toruloides was investigated by reintegrating pGPD1-, pFBA1-, pPGK1-, pPGK1, and pTFE1-driven Cas9 cassettes to a single clone of R. toruloides containing the expression cassette 55-tRNA-CRTYB Guide 1. While pGPD1 and pPGK1 showed editing activity, even with the now-optimized gRNA expression system, the gene editing levels observed for pTFE1, pFBA1, and pPGI remained <1%. Conversely, the pGPD1- and pPGK1-Cas9 transformations yielded fewer colonies overall than the GFP negative control as well as the promoters with poor editing efficiency, even though the effect was much more pronounced for pGPD1 while pPGK1-Cas9 yielded nearly as many colonies as the ineffective promoters and control despite similar editing efficiency to pGPD1 (Table S6). These data suggest the double-strand breaks induced by Cas9 in R. toruloides lead to significant cell death among the transformed population, and that this cytotoxicity is correlated (although not perfectly) with the degree of editing achieved.

Finally, we attempted to knock out two genes in multiplex, which can significantly accelerate metabolic engineering work in an organism. CRTYB Guide 1 and LEU2 Guide 1 were expressed using two sequential 55-tRNA cassettes, which were integrated to the genome of an NP11 single clone already expressing pPGK1-Cas9-NLS3. Following initial work showing knockout only of the first gene in the sequence despite the confirmed activity of both guides individually, a 150 bp spacer of junk DNA was added between the gRNA cassettes. With this modification, a double-knockout rate of 78% was realized. Interestingly, the LEU2 knockout rate was 85% among white colonies (CRTYB-knockout) but only 10% among red (wild-type CRTYB; Table 2). This is likely due to the random nature of the Agrobacterium-mediated transformation. Integration of the double gRNA cassette to a less accessible genomic locus will result in lower expression of both gRNAs and thus lower Cas9 activity at both sites.

During the revision process of this study, a related work describing multiplexed CRISPR/Cas9 genome editing in R. toruloides was published (Otoupal et al., 2019). Following investigation of the S. cerevisiae and Y. lipolytica SNR52 promoters, as well as R. toruloides tRNA genes, single and multiplex (double) knockouts were achieved at maximum rates of 46.2 ± 22.2% and ~0.3%, respectively, using a tRNA gene as a gRNA promoter to knock out CRTYB (also known as CAR2) and URA3. These results highlight that the use of tRNA genes as a promoter to drive gRNA expression can be somewhat effective in R. toruloides, but the 5S-tRNA fusion strategy seems to allow for significantly higher editing efficiencies in this yeast by increasing the gRNA transcript abundance.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Knockout efficiency</th>
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<tbody>
<tr>
<td>CRTYB</td>
<td>95 ± 1%</td>
</tr>
<tr>
<td>LEU2 (CRTYBΔ)</td>
<td>85 ± 7%</td>
</tr>
<tr>
<td>LEU2 (CRTYB WT)</td>
<td>10 ± 0%</td>
</tr>
<tr>
<td>LEU2, overall</td>
<td>82 ± 7%</td>
</tr>
<tr>
<td>Double KO</td>
<td>78 ± 7%</td>
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Note: Error represents standard deviation of biological duplicates.

Abbreviations: gRNA, guide RNA; tRNA, transfer ribonucleic acid.
laid a solid foundation for achieving these outcomes and should open the door for much future engineering work of this organism.

2 | MATERIALS AND METHODS

2.1 | Strains and media

*R. toruloides* strain NP11 (a haploid strain derived from the diploid strain Y4) was used for CRISPR/Cas9 gene editing experiments. Once the Cas9 expression design pPGK1-Cas9-NLS3 was finalized and integrated to NP11 by *Agrobacterium tumefaciens*-mediated transformation (ATMT), a single clone of this strain was used for all subsequent gene editing experiments. The yeast was grown at 30°C, 250 rpm in YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose). As appropriate, the YPD media was supplemented with 200 μg/ml G418 (KSE Scientific, Durham, NC) or 50 μg/ml hygromycin (Gold Biotechnology, Olivette, MO) for selection.

A. *tumefaciens* strain AGL1 was grown at 30°C, 250 rpm in Luria Broth (LB) medium. Kanamycin (50 μg/ml) was used for selection. Cloning was performed in *Escherichia coli* strain NEB 10β (New England Biolabs, Ipswich, MA). Induction media (IM) plates for performing ATMT were prepared as described previously with 200 μM acetosyringone (Beijersbergen & Hooykaas, 1995). For knockout of LEU2, the induction media was supplemented with 100 mg/L leucine. Screening of LEU2 knockouts was performed on SC-L (6.7 g/L YNB without amino acids; BD Difco, Franklin Lakes, NJ; 0.69 g/L CSM-Leu; MP Biomedicals, Santa Ana, CA, and 20 g/L glucose) plates with SC plates (SC-L supplemented with 100 mg/L leucine) as a positive control.

2.2 | Plasmid construction

All plasmids used were derived from ATMT plasmids provided by Zhongbao Zhao and Stephen Aves’s laboratories. The RNAP III gRNA expression helper plasmids NM1-tRNA-SgH, NM1-5S-SgH, and NM1-5S-trRNA-SgH were created by digesting pZPK-PGPD-HYG-Tnos (modified to be BbsI-free by introducing one point-mutation using PCR) with EcoRI and KpnI and ligating in the desired gRNA expression cassette with two BbsI sites in the promoter and polyT terminator for subsequent cloning of the gRNA sequence. These helper plasmids were then digested with BbsI and the 20-nucleotide guide sequences created by phosphorylating and annealing two complementary oligos with appropriate overhangs which were then ligated into the backbone.

The RNAP II and RNAP II/III gRNA expression helper plasmids NM8-HDV-SgH and NM8-5S-trRNA-5S-TrNA-SgH were created by digesting pEGFP-Rt-YR-HYG (with the backbone modified to be BsaI-free by introducing five point mutations using PCR and GoldenGate assembly) with PmlI and SpeI and cloning in the desired gRNA expression cassette ordered as a gBLOCK with two BsaI sites for subsequent cloning of the gRNA sequence (Integrated DNA Technologies, Coralville, IA) using DNA assembler (in-yeast assembly) as described previously (Shao, Zhao, & Zhao, 2009; Johns et al., 2016). These helper plasmids were then digested with BsaI and the 20-nucleotide guide sequences created by Golden-Gate assembly with a Hammerhead-gRNA gBLOCK (for NM8-HDV-SgH) or by phosphorylating and annealing two complementary oligos with appropriate overhangs which were then ligated into the backbone (for NM8-5S-trRNA-5S-TrNA-SgH).

The Cas9 expression plasmid NM9-SpCas9-NLS3 was created by digesting pEGFP-Rt-YR-G418 with PmlI and SpeI and inserting a codon-optimized SpCas9-NLS3 gene (GenScript) using DNA assembler (Shao et al., 2009). The NLS sequences SV40 and NLS3–7 were ordered as gBLOCKs (replacing the blunt PmlI site with MfeI) and inserted at the N-terminus of Cas9 by linearizing NM9-pPGK1 SpCas9-NLS3 with PmlI and performing another in-yeast assembly. The PGK1 promoter expressing Cas9 was switched for pGPD1, pFBA1, pPGI1, and pTEF1 by digesting NM9-pPGK1-SV40-SpCas9-NLS3 with AflII and MfeI, pGPD1, pFBA1, and pPGI1 were PCR amplified from the plasmids pZPK-PGPD-HYG-Tnos, pZPK-PFBA-HYG-Tnos, pZPK-PPGI-HYG-Tnos, and *R. toruloides* NP11 genomic DNA, respectively, with the appropriate restriction sites added and ligated into the backbone. Primers and plasmids used in this study are listed in Tables S7 and S8, respectively.

2.3 | Transformation of *R. toruloides*

Transformation of *R. toruloides* was performed using ATMT as described previously (Lin et al., 2014). Briefly, overnight cultures of the *R. toruloides* strain to be transformed and the *A. tumefaciens* strain carrying binary plasmid with the desired genetic material were grown in YPD (with appropriate antibiotic, if necessary) and LB/kanamycin, respectively. 1.8×10⁷ cells were collected and washed once with sterile water and resuspended in 1 ml of sterile water. One hundred microliters each of *R. toruloides* and *A. tumefaciens* were mixed and spread onto an IM plate and incubated at 25°C for 48 hr. Cells were then recovered from the plate with 1 ml of sterile water, centrifuged and resuspended in 200 μl of sterile water. One hundred microliters of cells was then plated onto selective YPD media and allowed to grow for up to 5 days (until colonies appeared and were large enough for genotyping).

2.4 | Screening of CRTYB and CRT1 knockout mutants

Following ATMT of Cas9 and the appropriate gRNA, *R. toruloides* colonies were grown on YPD/G418/hygromycin until the majority of the colonies had reached approximately 2 mm in diameter (3–5 days). In our experience, the red pigmentation is still very faint at this point, so the plates were then kept at 4°C until the red color became more pronounced, after one to two weeks.

2.5 | Screening of LEU2 knockout mutants

Following ATMT of Cas9 and the appropriate gRNA, ten *R. toruloides* colonies were picked from each plate and suspended in 10 μl of sterile water. Three microliters of the suspension were spotted onto
SC and SC-L plates, which were incubated at 30°C for 2 days. Knockout rate was calculated as the proportion of colonies which grew on SC but not on SC-L media.

2.6 | Quantitative polymerase chain reaction

*R. toruloides* cultures were inoculated from a plate and grown for 24 hr in YPD supplemented with appropriate antibiotic and then recultured from a starting OD of 0.1 and grown to mid-log phase (OD 2–3). Total RNA was extracted using the Qiagen RNeasy kit (Qiagen, Venlo, The Netherlands) and reverse transcription was performed with Bio-Rad iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA), with a prior 10 min, 65°C denaturation step performed to disrupt gRNA secondary structure. qPCR was performed using Bio-Rad iTaq Universal SYBR Green Supermix on a Roche Lightcycler 480 qPCR system. Phosphoglycerate kinase was used as the reference gene for relative quantification.

2.7 | Genomic DNA extraction

Genomic DNA extraction from *R. toruloides* was performed using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI).

2.8 | Identification of NLS sequences

Endogenous NLS sequences were identified using cNLS Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) to identify potential NLS sequences within endogenous putative nuclear proteins.

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CONFLICT OF INTERESTS

The authors declare the following competing financial interest(s): J. C. S., M. C., and H. Z. declare competing financial interest in the form of a pending patent application.

ORCID

Huimin Zhao  http://orcid.org/0000-0002-9069-6739

REFERENCES


**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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