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Standardized Markerless Gene Integration for Pathway Engineering in *Yarrowia lipolytica*

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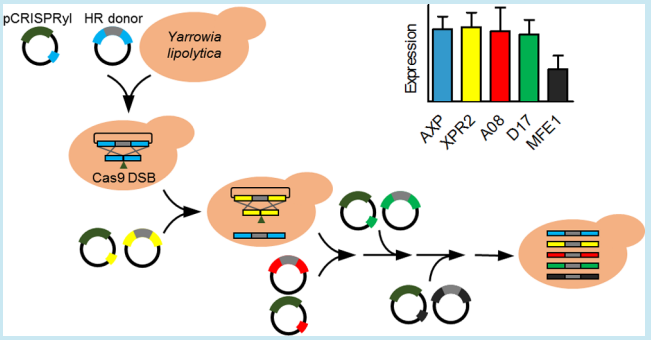
Supporting Information

ABSTRACT: The yeast *Yarrowia lipolytica* is a promising microbial host due to its native capacity to produce lipid-based chemicals. Engineering stable production strains requires genomic integration of modified genes, avoiding episomal expression that requires specialized media to maintain selective pressures. Here, we develop a CRISPR-Cas9-based tool for targeted, markerless gene integration into the *Y. lipolytica* genome. A set of genomic loci was screened to identify sites that were accepting of gene integrations without impacting cell growth. Five sites were found to meet these criteria. Expression levels from a GFP expression cassette were consistent when inserted into AXP, XPR2, A08, and D17, with reduced expression from MFE1. The standardized tool is comprised of five pairs of plasmids (one homologous donor plasmid and a CRISPR-Cas9 expression plasmid), with each pair targeting gene integration into one of the characterized sites. To demonstrate the utility of the tool we rapidly engineered a semisynthetic lycopene biosynthesis pathway by integrating four different genes at different loci. The capability to integrate multiple genes without the need for marker recovery and into sites with known expression levels will enable more rapid and reliable pathway engineering in *Y. lipolytica*.



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A valuable microbial host for the biosynthesis of lipid-based chemicals is the oleaginous yeast *Yarrowia lipolytica*.[1,2] A set of gene overexpressions and knockouts has been identified that creates strains that accumulate lipids to more than 90% of cell dry weight (CDW).[3−5] Synthetic and semisynthetic pathways have also been designed including pathways for the biosynthesis of omega-3 fatty acids, pentane, carotenoids, and α-ketoglutarate among others.[6−9] Its high capacity to produce and accumulate lipids has also made it a useful model for lipid metabolism.[10,11]

In comparison to the model yeast *Saccharomyces cerevisiae*, *Y. lipolytica* has been more difficult to engineer. The high capacity of *S. cerevisiae* to undergo homologous recombination (HR) is the basis of many advanced synthetic biology and genetic engineering tools.[12−14] HR in *Y. lipolytica* is significantly reduced and the number of advanced tools is relatively limited.[15,16] Integrative transformation and marker recovery by Cre-Lox recombination[17] is available as are a number of stable expression plasmids,[18] but genome integrations occur at low efficiency and have relied on selectable markers.[19,20] We recently added to the available genome engineering tools by developing a CRISPR-Cas9 system for use in *Y. lipolytica*,[21] but the lack of advanced synthetic biology tools has limited rapid strain development and targeted markerless gene integration, which is favored in industry,[22−24] is still challenging.

Overexpression from chromosomal genes has been shown to be more consistent across cell populations than expression from episomal genes.[25,26] Targeted and random gene insertions into *S. cerevisiae* have also revealed that expression can vary upward of 9-fold between genes integrated at different loci.[27,28] Similar effects have been observed in *Escherichia coli* and *Bacillus subtilis*, where gene expression has been shown to be higher from loci closer to the origin of replication.[29,30] The discovery of the type II CRISPR-Cas9 system from *Streptococcus pyogenes* and its adoption for genome editing has made less genetically tractable organisms more accessible and promises to enable more genome-wide and context-dependent expression studies.[21,31,32]

In this work, we developed a tool for markerless gene integrations into the *Y. lipolytica* genome at well-characterized sites. Using HR to repair CRISPR-Cas9 created double stranded breaks, we identified five loci where gene integration occurred at reasonably high efficiency (AXP, XPR2, A08, D17, and MFE1; Table 1). Experimental characterization of strains with an integrated model GFP expression cassette showed that disruption of the identified sites did not affect cell growth and that expression varied across selected sites. Finally, we

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Table 1. Description of Each Site Used as an Integration Target

site	description
AXP	acid extracellular protease, knocked out in PO1f strain
XPR2	alkaline extracellular protease, knocked out in PO1f strain
A08	pseudogene, no known function
D17	pseudogene, no known function
MFE1	multifunctional enzyme 1, essential for β-oxidation

demonstrated the utility of the gene integration tool by engineering a lycopene biosynthesis pathway in the *Y. lipolytica* genome without integrating selectable auxotrophic markers.

To identify suitable genomic loci, we screened 17 unique sites for CRISPR-Cas9-mediated HR of a heterologous humanized *Renilla* green fluorescent protein (hrGFP) expression cassette (Figure 1). The set of loci included four sites in cryptic sugar metabolism genes,[20,33] five sites in β-oxidation genes,[7,34] and five sites in annotated pseudogenes (Table S1).[35] Three additional sites, AXP, XPR2, and LEU2, were screened as they are functionally disrupted in the PO1f strain. The β-oxidation genes (POX2, POX3, POX4, POX5, and MFE1) were selected because they are often disrupted when engineering high lipid accumulation,[4,7,34,36] the pseudogenes (A08, A11, B20, D17, and E07) were selected because they are annotated as not coding for functional protein products, and the cryptic sugar metabolism genes (XDH, XLK, XYR, and GAL10) were selected because they are not necessary for growth on glucose or lipid feedstocks.[20,33]

For each site, a homology donor plasmid was constructed with an hrGFP expression cassette flanked by 1 kb homology up and downstream of the targeted Cas9 cut site (Figure 1A, B). The donor plasmids were designed so that the targeted protospacer adjacent motif (PAM) was eliminated after recombination, thus avoiding Cas9 retargeting. Unique restriction sites between the homology regions were included for simple cloning with new genes of interest (Figure S1). This enables the generation of plasmids for integrating any gene of interest with only a single restriction digest and ligation. A second plasmid (pCRISPRyl) expressed codon optimized Cas9 from *S. pyogenes* and a targeting sgRNA.[21,37] sgRNAs sequences were designed using a previously defined scoring algorithm,[38] with the highest scoring sgRNAs per gene selected for use. These sequences were designed to match the PO1f genome, which has high similarity but is not identical to the genomes of other *Y. lipolytica* strains.

HR rates in *Y. lipolytica* using selectable genetic markers are reported to vary between 2 to 44%.[15−17] Given the reported success of CRISPR-Cas9-mediated gene integration in *S. cerevisiae* and other yeasts,[23,39−41] we hypothesized that Cas9-induced double stranded breaks in the *Y. lipolytica* genome would increases HR rates and eliminate the need for integrated genetic markers. Figure 1D shows selected screening results of hrGFP integration into the AXP site. Gene integration was achieved by cotransforming pCRISPRyl and a HR donor plasmid specific to AXP. Two days of outgrowth in double selective media (LEU$^-$ and URA$^-$) followed by plating on rich media produced isolated colonies. Screening for integrations was accomplished using a three-primer colony PCR. A 2 kb PCR product indicated the PO1f genotype, while a 1 kb PCR product resulted from the integration of the hrGFP expression cassette. In the selected examples shown in Figure 1D, three of six screened colonies showed successful integrations.

Of the 17 tested loci, five resulted in efficient heterologous gene integration. MFE1 showed the highest efficiency at 69 ± 25%. Integration into AXP occurred with an efficiency of 62 ± 10%, while XPR2, A08, and D17 produced integrations with efficiencies of 48 ± 13%, 53 ± 33%, and 52 ± 13% (Figure 2A, Table S2). Three additional sites (POX4, E07, and XDH) showed integrations with efficiencies less than 6%, while no integrations were observed in the other nine tested sites. It is possible that these sites were less amenable to gene integration

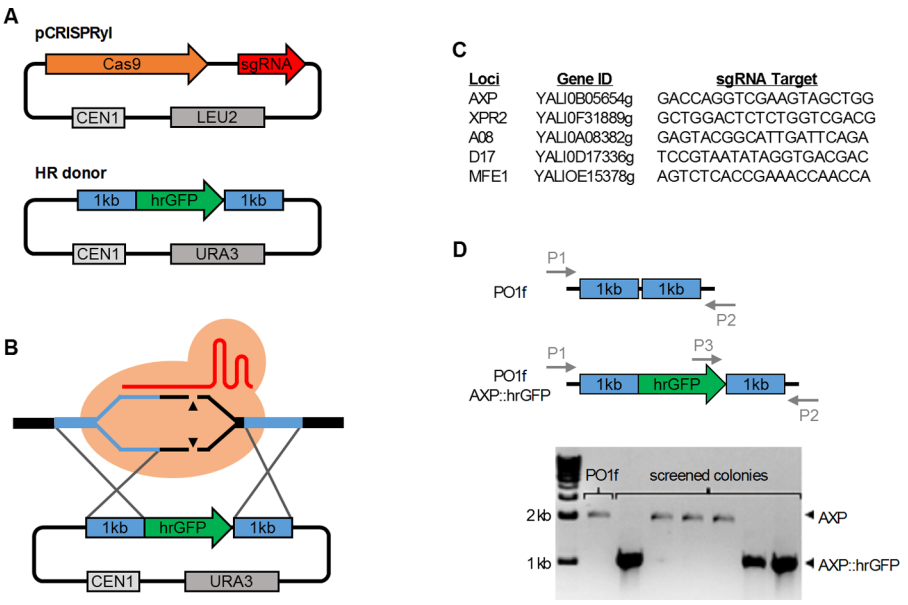

Figure 1. System for markerless gene integration in *Y. lipolytica*. (A) CRISPR-Cas9 expressing and homology donor plasmids. (B) Schematic representation of CRISPR-Cas9 induced homologous recombination. (C) Selected genomic loci with *Y. lipolytica* CLIB122 annotation and sgRNA targeting sequences. (D) Examples of hrGFP cassette integration into AXP including a schematic of PCR-based screening (top) and electrophoretic gel (bottom).

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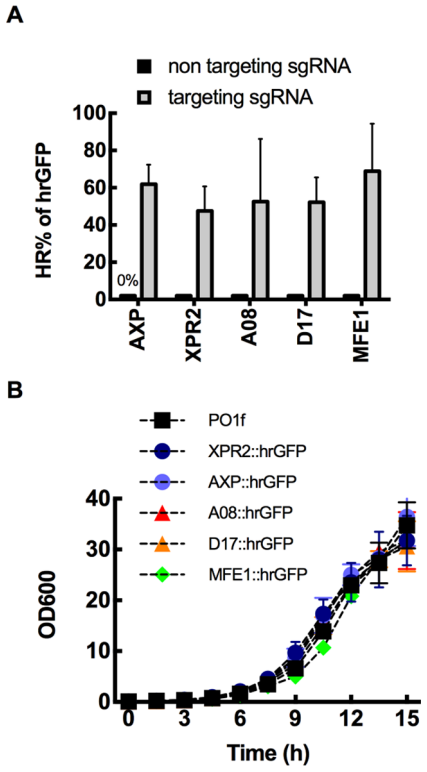

Figure 2. Targeted genome integration sites in *Y. lipolytica*. (A) Homologous recombination (HR) efficiency of an hrGFP expression cassette into AXP, XPR2, A08, D17, and MFE1. Each site was tested with and without a targeting sgRNA. Integration rates are the average of three biological replicates with at least a total of 24 colonies screened across all replicates. (B) Growth rates of strains with hrGFP integrated into the indicated site. Growth curves are the average of three separate integrated colonies grown on rich media (YPD).

due to DNA accessibility. Previous work has shown that chromatin structure and nucleosome occupancy influence the efficiency of CRISPR-Cas9-mediated processes in mammalian cells, this may also be the case in *Y. lipolytica* and other yeasts.[42] In addition, no integrations were observed in control experiments with nontargeting sgRNAs and in the absence of the pCRISPRyl plasmid showing that the targeted Cas9 was necessary to induce integration (Figure 2A and Table S3). Disruption of nonhomologous end-joining (NHEJ) through knockouts of KU70 and KU80 has previously been shown to increase HR efficiency in *Y. lipolytica*.[15,16] As such, we tested our system in a KU70 disrupted background. In the absence of NHEJ, integrations into LEU2, XLK, and XYR were possible, increasing HR efficiency from 0 to between 7 and 28% (Figure S2). In the case of the five high efficiency integration sites, KU70 disruption had little to no effect. In a previous work, it was found that disruption of KU70 resulted in an increase from 74% to 100% at a site within the MFE1 gene.[21]

Colonies that showed positive integrations of hrGFP into AXP, XPR2, A08, D17, and MFE1 were cured of the two-plasmid system by overnight growth in rich media supplemented with 5-fluoroorotic acid (5-FOA). Plasmid removal was confirmed by an inability to grow in both URA$^-$ and LEU$^-$ medium (PO1f is an engineered URA and LEU auxotroph). Growth studies in rich media revealed that hrGFP integration into each of the high efficiency sites had no significant effect on growth (Figure 2B).

The developed experimental protocol allows for the integration of a single gene in 5 days. The protocol includes 2 days of outgrowth in URA$^-$/LEU$^-$ media after transformation, 1 day to produce colonies on solid rich media (for colony PCR-based screening), 1 day for plasmid removal in liquid culture, and 1 day to isolate colonies with successful integrations. A reduced time protocol of 4 days was also achieved by screening for gene integration after plasmid removal. In this case, after 2 days of outgrowth in URA$^-$/LEU$^-$ selective liquid media a small volume of the liquid culture was used to inoculate rich liquid media containing 5-FOA to cure the plasmids. Plating on rich media produced isolated colonies that can be screened for successful integrations with pCRISPRyl and the HR donor plasmid already removed. Schematics of the protocols are shown in Figure S3.

To quantify UAS1B8-TEF(136) driven expression of hrGFP, the integrated strains were analyzed by flow cytometry during midexponential and stationary phase (Figure 3A). For all five

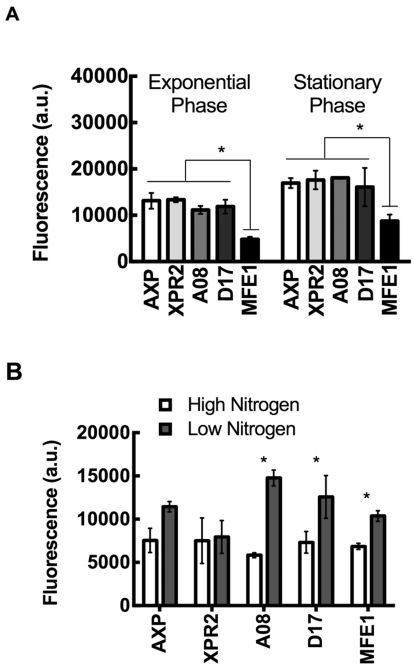

Figure 3. Quantification of hrGFP expression from *Y. lipolytica* genomic loci. Flow cytometry quantification of hrGFP fluorescence at midexponential and stationary phase from each site on rich media (A) and synthetic media with high and low nitrogen content at stationary phase (B). Statistical significance indicated with * for p values <0.01. With the exception of AXP, all experiments were performed on three biological replicates (AXP, n = 2).

sites, expression was higher at stationary phase than during exponential phase (exponential to stationary phase effect, $p < 0.01$). During exponential growth, there was no statistical difference in hrGFP expression across AXP, XPR2, A08, and D17, but expression from MFE1 was significantly reduced ($p < 0.01$). Similarly, expression from MFE1 at stationary phase was reduced in comparison to the other sites ($p < 0.01$). Studies of heterogeneous expression from *S. cerevisiae* genomes have shown significantly higher variation between loci and a previous study in *Y. lipolytica* showed upward of a 2.7-fold difference in expression for an integrated expression cassette.[27,28,43] Here, only MFE1 was found to differ from the other characterized sites and the effect was limited to a ~2.3-fold reduction in

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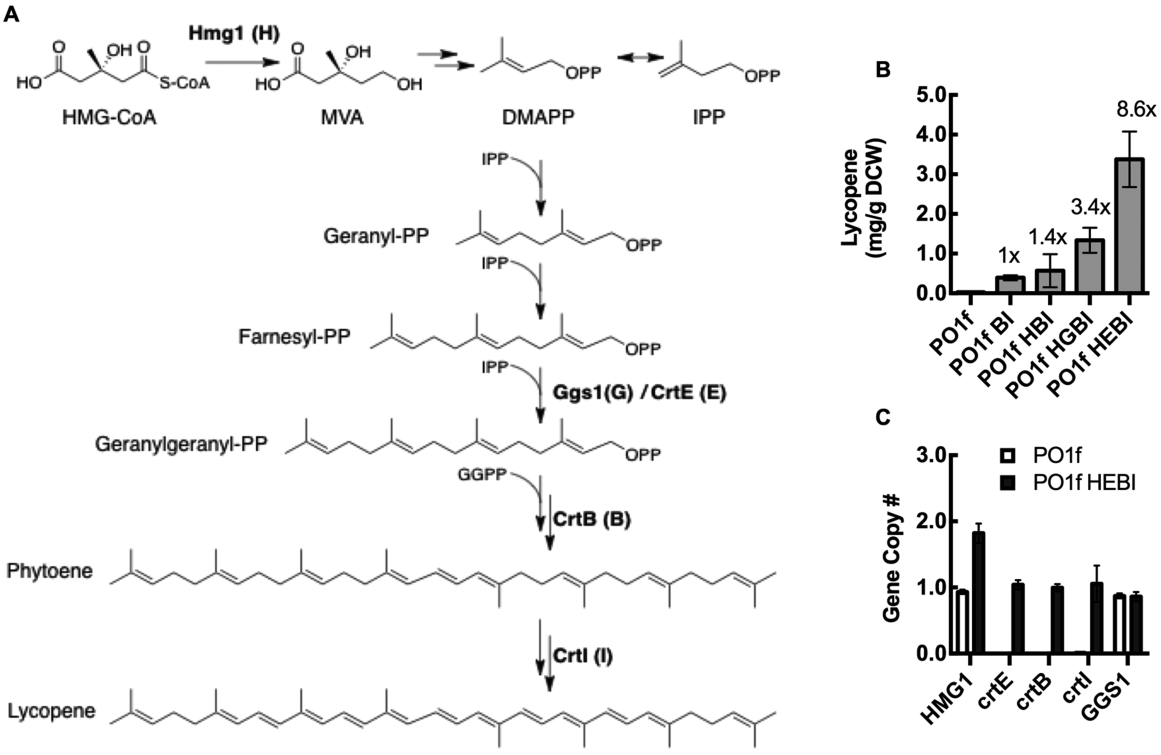

Figure 4. Lycopene biosynthesis pathway engineering. (A) The semisynthetic lycopene biosynthesis pathway in *Y. lipolytica*, including both upstream mevalonate pathway and heterologous lycopene production genes. Integrated genes include HMG-CoA reductase (HMG1), GGPP synthase (GGS1 and crtE), phytoene desaturase (crtI) and phytoene synthase (crtB). (B) Production of lycopene from generated strains grown on rich media with 10% glucose in shake flasks after 4 days of culture. Measurements represent the average and standard deviation of three biological replicates. (C) Quantification of copy number of each gene in the PO1f and the PO1f HEBI strains, as measured by qPCR on isolated genomic DNA. Measurements represent at least three biological replicates. HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; MVA, mevalonic acid; DMAPP, diemthylallyl pyrophosphate; IPP, isopentyl pyrophosphate; PP, pyrophosphate.

expression. As many *Y. lipolytica* studies rely on low nitrogen media to induce lipid synthesis and accumulation, we characterized expression from cultures in synthetic defined media with controlled nitrogen levels (Figure 3B). The AXP and XPR2 sites showed similar levels of expression when cells were grown on either high or low nitrogen media, while the A08, D17, and MFE1 sites showed increased expression in low nitrogen media ($p < 0.01$). As the same hrGFP expression cassette was integrated into each site, differences in expression are not likely to be due to the gene construct, but instead due to differences in the surrounding genomic environment produced with varying culture conditions.

To evaluate the suitability of the characterized sites for multigene pathway engineering, we integrated a synthetic lycopene biosynthesis pathway into PO1f. It has previously been shown that heterologous expression of phytoene desaturase (CrtI) and phytoene synthase (CrtB) is sufficient for lycopene production in *Y. lipolytica*.[8] CrtB catalyzes the condensation of two natively produced geranylgeranyl pyrophosphate (GGPP) molecules to phytoene, while CrtI converts phyoene to lycopene with four consecutive desaturation reactions (Figure 4A). *Y. lipolytica* codon-optimized *crtB* and *crtI* from *Pantoea ananatis* each driven by a UAS1B8-TEF(136) promoter were integrated into the AXP and XPR2 sites, respectively. The resulting strain, PO1f BI, produced 0.39 mg lycopene/g DCW in shake flask cultures with 10% glucose YPD over 4 days (Figure 4B).

In many yeasts, Hmg-CoA reductase (Hmg1) catalyzes the committed step of the mevalonate pathway and its over-expression has proven to be a useful strategy for the production of carotenoids.[44,45] Integration of a second copy of native HMG1 into the D17 site (PO1f HBI) produced 0.57 mg lycopene/g DCW in YPD10, a 1.4-fold increase over the PO1f BI strain. Further enhancements of lycopene biosynthesis were possible with the overexpression of GGPP synthase (Ggs1), which catalyzes the condensation of farnesyl pyrophosphate and isopentenyl pyrophosphate to GGPP. Integration of a second copy of the native GGPP synthase, GGS1, into the A08 site (PO1f HGBI) resulted in 1.34 mg lycopene/g DCW, while integration of a heterologous ortholog from *P. ananatis*, *crtE* (PO1f HEBI, Figure S4), produced 3.38 mg lycopene/g DCW, representing 3.4- and 8.6-fold increases in lycopene production over the PO1f BI strain, respectively.

A recent study demonstrated heterologous gene duplication during the lab evolution of a high xylose utilizing strain of *Y. lipolytica*.[46] Duplication of genes in the synthetic lycopene pathway would likely affect pathway flux and our analysis of the standardized integration system; as such, we quantified gene copy number in PO1f HEBI, the highest producing strain. Quantitative PCR revealed gene copy numbers that matched our expectations: PO1f HEBI had single copies of the heterologous *crtB*, *crtE*, and *crtI*, a single copy of native GGS1, and two copies of HMG1, one native copy and a second copy integrated into the D17 site (Figure 4C).

It is important to note that integrations of HMG1, GGS1, and *crtE* occurred at lower efficiencies than the integration of hrGFP into the same sites. Overexpression of the synthetic pathway resulted in increased lycopene biosynthesis, which has

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previously been shown to negatively affect growth rate.[47−49] In our standardized integration system, outgrowth in liquid culture was used to reduce the total protocol time for gene integration, with only 4 days being needed per gene. In this context, mutants with growth defects (*e.g.*, cells producing lycopene) were outcompeted by cells with faster growth rates resulting in low integration efficiency. The decreased integration efficiency may have also been due to the larger size of the lycopene pathway genes in comparison to hrGFP. To overcome this challenge, cells were plated on selective media immediately following transformation and mature colonies were screened for integrations. For integration of HMG1, GGS1, and *crtE* into strains containing *crtB* and *crtI* expression cassettes, between 10 and 50% of screened colonies showed partial integration. Single colonies showing partial integration were then isolated *via* restreaking to solid rich media, rescreened to confirm integration, and subjected to plasmid removal. This protocol adds to the total time for a single integration because plating immediately after transformation on selective media results in some heterogeneous colonies (*i.e.*, gene integration could occur during colony growth in some cells but not others). The process of plating on selective media (3 days for mature colonies), isolating single colonies by restreaking on rich media (1 day), growth in rich liquid media with 5-FOA for plasmid removal (1 day), and plating for single colonies (1 day) results in a total of 6 days per gene integration. This strategy was used to generate strains PO1f HBI, PO1f HGBI, and PO1f HEBI.

Integration into *Y. lipolytica* genome has thus far relied on selectable genetic markers to identify successful homology-targeted or random insertions. For example, the Cre-Lox systems has been used to perform sequential integrations and recycle available genetic markers,[17] multiple copies of a single heterologous gene are commonly inserted into repeat rDNA and zeta sites by HR or random integration using a defective URA3 marker,[18,43,50] and multigene cassettes have been integrated across the genome with selectable markers.[51] Multigene integration has also been achieved by transforming *Y. lipolytica* with multiple overlapping linear DNA fragments that recombine and integrate into the genome with the assistance of a selectable genetic marker.[52] By leveraging targeted double stranded breaks from CRISPR-Cas9, our system achieves high integration efficiencies of single genes over short time periods in comparison to these established methods. And importantly, the resulting gene insertions do not require a genome-integrated selectable marker. Similar systems have been developed for *S. cerevisiae*, reaching efficiencies as high as 100% for single gene integrations, and allowing for multiplexed gene integration.[22,24,40,41] These systems benefit from the high capacity of *S. cerevisiae* to undergo HR, a phenotype not present in *Y. lipolytica*. Multiplexed gene disruption has been achieved in *Y. lipolytica* using CRISPR-Cas9, and so multiplexed gene integrations may be possible using a similar system.[32] Here, we found that HR efficiencies upward of 50% are possible in *Y. lipolytica* without disrupting DNA repair mechanisms (*e.g.*, NHEJ) in selected genomic loci. Interestingly, in 6 of the 17 screened sites we were not able to achieve homologous recombination.

In this work, we developed a system of markerless gene integration into predefined genomic loci in *Y. lipolytica*. By using CRISPR-Cas9-induced HR, a heterologous expression cassette can be integrated in a minimum of 4 days, with no need for marker recovery. Genome loci amenable to integration were identified and the gene integration tool was expanded to include five pairs of plasmids, with each pair design to target a unique site with high efficiency. Importantly, the homology donor plasmid was designed for easy cloning of any gene of interest. Expression from each identified genomic loci (AXP, XPR2, A08, D17, and MFE1) was quantified and characterized. The set of standardized sites could be expanded to include repetitive genomic sequences from rDNA and zeta site loci, thus enabling multigene integration in a single step. By standardizing a system for integration of heterologous genes in *Y. lipolytica*, we were able to express and improve a synthetic lycopene biosynthetic pathway.

METHODS

Strains and Media. The PO1f strain of *Yarrowia lipolytica* (*MatA, leu2−270, ura3−302, xpr2−322, axp-2*)[53] (ATCC no. MYA-2613) was used in all genome editing and expression experiments in this study. Cultures were grown in YPD medium (1% Bacto yeast extract, 2% Bacto peptone, 2% glucose) or synthetic defined (SD) media without leucine and uracil (0.67% Difco yeast nitrogen base without amino acids, 0.067% CSM-Leu-Ura (Sunrise Science, San Diego, CA), and 2% glucose) at 30 °C at 200 rpm in 14 mL tubes. Growth curves of *Y. lipolytica* were done in YPD media by inoculating 25 mL to an initial OD600 of 0.05 in a baffled 250 mL flask and incubating at 30 °C at 200 rpm. Samples were taken at regular time points and the OD600 measured using a NanoDrop 2000 UV−vis Spectrophotometer. DH5α *Escherichia coli* was used for plasmid construction. *E. coli* was grown in LB medium with 100 mg/L ampicillin at 37 °C. Yeast strains used and generated in this study are shown in Table S4.

Cloning Procedures. CRISPR-Cas9 plasmids targeting genomic loci were generated using pCRISPRyl as previously described using Gibson Assembly of annealed oligonucleotides.[21,54] All oligonucleotides are shown in Table S5, and sgRNA target sequences are shown in Figure 1 and Table S1. Target sequences were selected by identifying the highest scoring unique sequences with a previously developed sgRNA scoring algorithm.[38]

Homology donor plasmids were generated from pUAS1B8-TEF(136)-hrGFP.[55] The LEU2 marker was replaced with the *Y. lipolytica* URA3 marker, and the resulting backbone was then amplified by PCR and assembled with 1kb homology arms using Gibson Assembly. SpeI and AvrII restriction sites were added between the homology regions. The UAS1B8-TEF(136)-hrGFP-CYC1t cassette was then inserted between the homology arms using SpeI and AvrII digestion and ligation. Homology arms were designed so that successful recombination into the genome would result in loss of the PAM sequence and between 3 and 5 additional bases. Selection of integration loci and design of homology regions was guided by the CLIB122 annotated genome, and sequences were designed based on published genomes for the PO1f strain and its parental strain.[35,56,57] Homology donor plasmids and pCRISPRyl plasmids have been deposited at Addgene (Plasmids #84608−84617).

Heterologous genes in the lycopene pathway (*crtB*, *crtE*, and *crtI*) were codon-optimized using Optimizer[58] and ordered as gBlocks from IDT with BssHII and NheI sites at the 5′ and 3′ ends, respectively. Sequences of codon-optimized genes are available in Table S6. The HMG1 and GGS1 genes were amplified from *Y. lipolytica* genomic DNA with BssHII and NheI sites at the 5′ and 3′ ends, respectively. All genes were cloned into homology donor plasmids using BssHII and NheI

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digestion and T4 DNA ligase for ligation or Gibson Assembly. Plasmids used in this study are shown in Table S4.

Genomic Integrations. *Y. lipolytica* transformations were performed using a lithium acetate protocol as previously described.[21] Approximately 0.5 μg of each plasmid was used in each transformation, with dual plasmid transformations yielding 15 to 100 colonies. Three variations of outgrowth and screening methods are illustrated and described in Figure S3. YPD media supplemented with 1 mg/mL 5-FOA was used for URA3 marker plasmid removal. Outgrowth in rich media with no counter selection was found to be effective for curing the LEU2 marker containing pCRISPRyl plasmid. Screening for integration was accomplished using colony PCR of single colonies added directly to the PCR reaction as shown in Figure 1.

Flow Cytometry. Transformants from glycerol stocks were streaked out on YPD plates and allowed to grow for 1 day. A single colony from each plate was used to inoculate 2 mL YPD liquid cultures in 14 mL culture tubes. Once visible growth was observed, cell densities of precultures were measured and cells were then passaged into fresh YPD media at a starting OD600 of 0.1. Cells were grown to exponential phase (OD600 of ∼35) and then stationary phase (OD600 of ∼60) prior to harvesting for flow cytometry analysis. For controlled nitrogen level experiments, cells from YPD precultures were passaged into Yeast Synthetic Complete (YSC) media (Sunrise Science, San Diego, CA). A high nitrogen condition was created by supplementing YSC with 5 g/L ammonium sulfate. YSC without the addition of ammonium sulfate was used for the low nitrogen condition. The cultures were inoculated at a starting OD600 of 0.2. High nitrogen condition cultures were grown for 48 h prior to flow cytometry analysis, while the low nitrogen cultures were grown for 72 h due of slower growth rates under nitrogen depleted conditions.

Prior to analysis, the cells were spun down at 6000*g* for 1 min, washed once and resuspended in 0.1 M phosphate buffered saline (PBS) solution (Sigma-Aldrich). The BD accuri C6 flow cytometer was used for data collection and analysis. A control cell population that was not expressing GFP was first run to identify basal cell autofluorescence prior to collecting data for the experimental samples. For each sample, 20 000 events were collected and the Virtual Gain analysis software was used to normalize gains relative to the control across all experimental samples post data collection. All experiments were performed in biological triplicate.

Lycopene Quantification. Cultivation of carotenoid producing strains was conducted in 250 mL shake flasks. Shake flasks containing 25 mL of a 10% glucose YPD medium were inoculated from overnight cultures to an OD600 of 0.1 and culture at 30 °C. Aliquots of the cultures were taken after 4 days of growth and used for lycopene quantification.

Extraction of carotenoids followed the method provided by Chen and co-workers with a few modifications.[59] A 5 mL sample was taken and used for DCW as previously described.[60] A 1 mL sample was used for extraction of lycopene by centrifuging at 5000*g* for 3 min, washing the cell pellet with water, resuspending in 1 mL 3 M HCl, and incubating at 100 °C for 2 min. Cells were then cooled in an ice bath for 3 min, washed with water, and resuspended in 1 mL of acetone. 200 μL of 500−750 μm glass beads (Fisher) were added, and the mixture was vortexed for 2 min. The mixture was then centrifuged and the supernatant was analyzed for lycopene. Lycopene was quantified by measuring absorbance at 472 nm and comparing to a standard curve created with purchased lycopene (Sigma; Figure S5).

Quantitative PCR. Genomic DNA was extracted from cells grown to stationary phase in YPD using the YeaStar genomic DNA kit from Zymo Research. Two μL of genomic DNA was subjected to qPCR using the SsoAdvanced Universal SYBR Green Supermix from Biorad on a CFX Connect thermocycler from Biorad. Primers were designed using Primer3 according to manufacturer's specifications,[61] and can be found in Table S5. Copy number was determined relative to actin using the amplification efficiency of each.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.6b00285.

Tested sites and integration rates with and without intact NHEJ. Plasmids, strains, primers, gene sequences. Schematic of integration protocols. Lycopene standards and images of strains producing lycopene. (PDF)

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Notes

The authors declare no competing financial interest.

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