

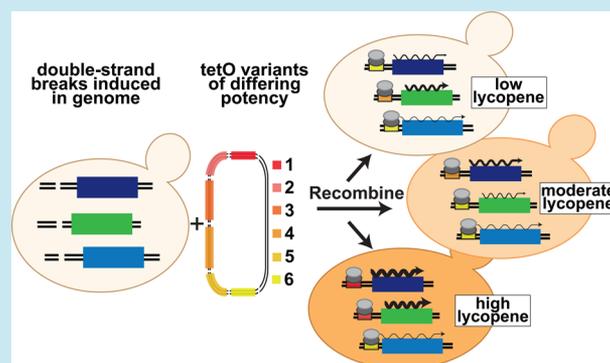
A tetO Toolkit To Alter Expression of Genes in *Saccharomyces cerevisiae*

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Supporting Information

ABSTRACT: Strategies to optimize a metabolic pathway often involve building a large collection of strains, each containing different versions of sequences that regulate the expression of pathway genes. Here, we develop reagents and methods to carry out this process at high efficiency in the yeast *Saccharomyces cerevisiae*. We identify variants of the *Escherichia coli* tet operator (tetO) sequence that bind a TetR–VP16 activator with differential affinity and therefore result in different TetR–VP16 activator-driven expression. By recombining these variants upstream of the genes of a pathway, we generate unique combinations of expression levels. Here, we built a tetO toolkit, which includes the I-OnuI homing endonuclease to create double-strand breaks, which increases homologous recombination by 10⁵; a plasmid carrying six variant tetO sequences flanked by I-OnuI sites, uncoupling transformation and recombination steps; an *S. cerevisiae*-optimized TetR–VP16 activator; and a vector to integrate constructs into the yeast genome. We introduce into the *S. cerevisiae* genome the three *crt* genes from *Erwinia herbicola* required for yeast to synthesize lycopene and carry out the recombination process to produce a population of cells with permutations of tetO variants regulating the three genes. We identify 0.7% of this population as making detectable lycopene, of which the vast majority have undergone recombination at all three *crt* genes. We estimate a rate of ~20% recombination per targeted site, much higher than that obtained in other studies. Application of this toolkit to medically or industrially important end products could reduce the time and labor required to optimize the expression of a set of metabolic genes.

KEYWORDS: genome engineering, yeast, tetO element, metabolic engineering, homologous recombination, lycopene



Many genome engineering efforts seek to optimize a microbial pathway for the production of a small molecule. In these approaches, the promoters of the genes encoding the enzymes of a biosynthetic pathway are modified so that the genes are expressed at different levels; the goal is to identify a strain with the best combination of expression levels that results in maximal production of the small molecule. Given suitable selection strategies, it is possible to examine on the order of thousands to millions of differing strains. In *Escherichia coli*, the modification of genes has been facilitated by technologies like multiplex automated genome engineering (MAGE), in which oligonucleotides are continually transformed and used to mutate promoters and ribosome binding sites.¹

However, many biological products are better produced in yeast. Engineered yeast has produced compounds that include fine chemicals such as terpenes, butanol, and other biofuels; protein drugs that include Insulin-like growth factor 1, immunoglobulin G, glucagon, and several protein components of vaccines; and drug precursors such as artemisinic acid.^{2–5} The yeast *Saccharomyces cerevisiae* is particularly well-suited for engineering, with its small, well-characterized genome and extensive libraries of gene deletions and plasmid-borne genes.

S. cerevisiae also has protein folding, proteolytic processing, glycosylation, and secretion pathways similar to those in mammalian cells.

S. cerevisiae is amenable to genome engineering because of its capacity for efficient homologous recombination, although its transformation efficiency is much lower than that of *E. coli*. The homologous recombination pathway can use DNA fragments whose ends have homology to genomic sites flanking a double-strand break to replace the endogenous sequence with altered versions. However, the rate of homologous recombination achievable in yeast has not been high enough to generate large numbers of strains, each with a unique set of expression levels of multiple genes. DiCarlo et al.⁶ improved yeast genome engineering by disrupting mismatch repair and overexpressing components needed for homologous recombination. Although their procedure increased recombination 500-fold, even after three rounds of transformation, genome modification had occurred at one locus in only 0.7% of cells and at two loci in

Received: December 12, 2014

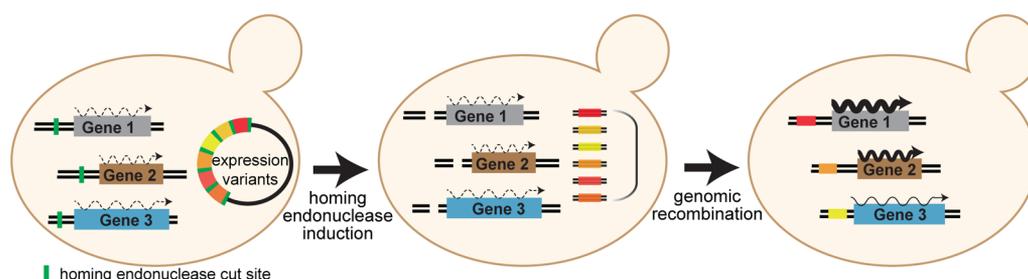


Figure 1. Overview of the tetO toolkit. To vary expression and bypass the low transformation efficiency of *S. cerevisiae*, we transform a plasmid that contains six versions of the tetO sequence with varying affinity for the Tet activator (left). Upon induction of a homing endonuclease, double-strand breaks occur both in the upstream regions of three genes targeted for modification and in the plasmid with the tetO variants (middle). Recombination occurs to insert one of the plasmid-borne tetO variants into each targeted gene, generating a library of *S. cerevisiae* colonies that differ in the amount of expression of these genes (right).

only 0.005%. Thus, a better method for rapidly generating changes in expression is needed.

An ideal method for yeast genome engineering that results in altered expression at several loci might encompass three critical features. First, it would replace the endogenous regulatory elements of target genes with known elements that result in differential expression levels. Second, it would increase homologous recombination near these elements by facilitating the generation of a double-strand break in each of the genes in the pathway being engineered. Third, it would bypass the need for iterative transformation by the use of a plasmid harboring multiple regulatory elements of variable strength, any of which can replace an existing element.

Here, we develop such a modular expression toolkit in *S. cerevisiae* using variants of the tet operator site (tetO) of *E. coli* as the binding site for a transcriptional activator, the Tet repressor–VP16 protein.⁷ We identified variants of this operator that generate over a 100-fold range of gene expression. By using combinations of these variants to drive expression of three genes needed for production of the antioxidant lycopene, we were able to select yeast whose high levels of lycopene derived from optimized expression of these genes. Furthermore, we developed a highly efficient homologous recombination process that allows us to rapidly swap tetO variants, resulting in strains with differing expression of targeted genes.

RESULTS

Overview of the tetO Toolkit. To increase the efficiency and ease of altering expression from several genes in *S. cerevisiae* at once, we built a set of genome engineering reagents. These include a collection of small, variant DNA elements that direct different levels of TetR–VP16-dependent gene expression. These elements were introduced into yeast by adapting the method of Wingler et al.,⁸ who overcame transformation deficiencies using plasmids that have previously been introduced into yeast to direct recombination. In their method, each recombination template was individually introduced on a single plasmid, thus requiring several rounds of transformation and recombination for strain construction involving multiple genes. In contrast, we combined several variant DNA elements in one plasmid, with each variant having the potential to recombine upstream of targeted pathway genes. To induce highly efficient recombination, we expressed a homing endonuclease and incorporated cut sites for it, both between the regulatory variants carried on the plasmid and upstream of the pathway genes. Double-strand breaks at these sites greatly increase recombination, resulting in unique combinations of

regulatory variants and consequent expression levels (Figure 1). Yeast that have undergone this process can be screened or selected for their production of an end product. Finally, our toolbox includes a vector to integrate genes; for example, a gene that carries an inactive tetO element can be integrated, with this element then replaced by one of the variant elements through recombination.

Optimization of Tet-Off Regulatory Control in *S. cerevisiae*. Most tetracycline-regulated activators are based on the *E. coli* Tn10 tetracycline resistance operon. This operon consists of divergently transcribed Tet repressor (*TetR*) and Tet resistance (*TetA*) genes separated by tet operator (tetO) sequences.⁹ In the presence of tetracycline, or its more stable derivative doxycycline (Dox), TetR changes conformation and disassociates from the tetO site. Although TetR is natively a repressor in bacteria, it was presumed that in eukaryotes TetR-based activators would more efficiently and specifically control expression.¹⁰ Thus, several Dox-responsive activators were developed containing the TetR protein fused to the VP16 transactivator of herpes simplex virus. If a TetR–VP16 fusion (Tet activator) drives expression in the absence of Dox, then the system is referred to as Tet-Off,^{7,11} whereas a variant activator that binds to the tetO site and drives expression in the presence of Dox is referred to as Tet-On.¹² Both systems are widely used in mammalian cell culture, *Drosophila*, zebrafish, and yeast.¹¹

We chose Tet-Off in order to carry out engineering without the necessity of including doxycycline for gene expression. To maximize the utility of the Tet-Off system in yeast, we sought to generate a strain producing the optimal amount of Tet activator. Because this activator is typically expressed under the control of a constitutive promoter, we first characterized the ability of five constitutive promoters commonly used in yeast to directly drive expression of the luciferase (*LUC1*) gene. Expression of *LUC1* can be readily determined by measuring luminescence after luciferase has acted on its substrate luciferin. Although the cytomegalovirus (CMV) promoter is often used in Tet-On and Tet-Off systems, this promoter, when directly activating the luciferase gene, led to low luciferase activity compared to that with four yeast promoters derived from the *CYC1*, *ADH1*, *TEF1*, and *TDH3* genes (Figure 2a).

We next tested how these five promoters behaved when they drove expression of the Tet activator, using a tetO:*LUC1* construct to assay activity indirectly. The CMV promoter driving the Tet activator again led to a low level of luciferase compared to that with the yeast promoters (Figure 2b and Supporting Information Figure S1a). The *CYC1* and *ADH1* promoters led

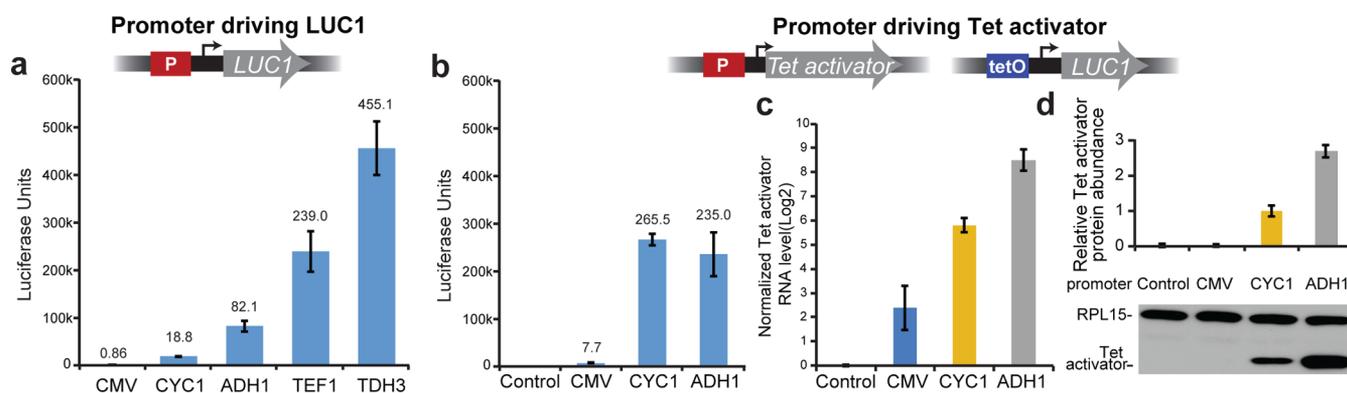


Figure 2. Optimization of the Tet-Off system in *S. cerevisiae*. (a) Mean (three biological replicates) \pm SE luciferase activity of the cytomegalovirus (CMV) and several yeast promoters to drive expression of the *LUC1* gene, as determined by luciferase activity. The CMV promoter drives extremely low expression, based on luciferase activity. (b) The CMV promoter and two yeast promoters were used to drive expression of the Tet activator gene. This activator, in turn, bound to the tetO site upstream of the *LUC1* gene such that luciferase activity reflects the amount of activator present; mean ($n = 3$) \pm SE luciferase activity. (c) Quantitative PCR was used to measure the amount of Tet activator transcript produced under the control of the three promoters. Mean (three biological replicates) \pm SE, normalized to *TAF10* mean expression. Tet activator expression was significantly higher when expression was driven from the *CYC1* or *ADH1* promoter. (d) Tet activator protein was determined by western blot using antibodies to TetR, and, as a loading control, the yeast Rpl15 protein was detected. Protein accumulation was quantified by spot densitometry; mean (three biological replicates) \pm SE.

to the greatest amount of luciferase, whereas both the *TEF1* and *TDH3* promoters produced less luciferase and more variable expression (Supporting Information Figure S1a). These latter two promoters, which are the strongest of the four tested¹³ (Figure 1a), also had severe effects on fitness of the yeast, with the *ADH1* promoter having a small effect on growth (Supporting Information Figure S1b,c). We compared the level of the Tet activator transcript (Figure 2c) and protein (Figure 2d) when this gene is under the control of either the *CYC1* or *ADH1* promoter. Expression of the Tet activator from the promoter of *CYC1* was optimal because it led to high luciferase activity from the tetO:*LUC1* reporter and no adverse effects on growth; this version of the Tet activator was used in all further experiments.

Variants of the tetO Site That Generate Different Expression Levels. We sought to identify tet operators with the potential for differential Tet activator-driven expression. The Tet activator is ideal for applying to end product optimization because the tetO binding site is small (19 nucleotides), well-characterized, and active in both prokaryotes and eukaryotes. The binding kinetics and action of TetR are also well-established and thus modifying the binding affinity of the tetO site for the Tet activator should result in a predictable reduction in activation. Mutations to tetO and TetR have been identified in *E. coli*,^{14,15} but the activity of tetO variants in *S. cerevisiae* has not been determined.

To identify tetO variants with differential activity in yeast, we built a *HIS3*-based reporter. Yeast lacking the His3 enzyme cannot survive without exogenous histidine, and His3 is sensitive to the competitive inhibitor 3-amino-1,2,4-triazole (3-AT). By replacing the regulatory elements of *HIS3* with a tetO site, we could measure *HIS3* expression by growth of yeast in the absence of histidine and presence of 3-AT or by quantitative PCR. The tetO variants were coupled to a minimal *CYC1* promoter, similar to other Tet activator schemes,¹⁶ and consisted of either a wild-type tetO site or a 4,14-C,G variant with low binding to TetR,¹⁴ and the variants were introduced upstream of the *HIS3* locus. The Tet activator was expressed from the *CYC1* promoter. Either version of the *HIS3* gene was sufficient for yeast to grow on media lacking histidine (Figure 3a),

indicating that the minimal *CYC1* promoter alone resulted in low baseline expression. However, only the wild-type tetO site resulted in enough Tet activator-driven expression to overcome 50 mM 3-AT (Figure 3a). Similarly, *HIS3* gene expression measured by qPCR was upregulated with the wild-type tetO compared to that with the 4,14-C,G variant (Supporting Information Figure S2a).

To identify tetO variants that alter Tet activator-directed expression, we used the *HIS3* reporter driven by the 4,14-C,G tetO variant. We transformed oligonucleotides into yeast corresponding to the wild-type tetO site doped at a 2.5% mutagenesis rate per nucleotide. If an introduced oligonucleotide recombined to replace the inactive tetO variant with a variant that binds better to the Tet activator, then we could detect this event as a colony that grew on media lacking histidine and containing 50 mM 3-AT. Sequence analysis of the tetO site in such colonies revealed several new tetO variants, which differed in how much growth they conferred in selective media (Figure 3b and Supporting Information Figure S2b). These variants also led to activities of the luciferase reporter similar to their effects in the *HIS3* reporter (Supporting Information Figure S2c). To quantify additional variants identified from the *HIS3* assay, we introduced the tetO variants into the luciferase reporter to assay activity relative to that of the wild-type tetO (Figure 3c). Among the variants tested, several maintained wild-type levels of luciferase activity, whereas others led to lower activity (Figure 3c). We chose variants with roughly 15–20% differences in expression from each other (Figure 3c, labeled in red), resulting in a 100-fold range of expression. We confirmed that this expression was specifically due to the Tet activator by also measuring luciferase activity when 10 μ g/mL doxycycline was added. All tetO variant-driven expression was still doxycycline-sensitive, indicating that it was due to Tet activator activity and not the formation of other upstream activation sites (Supporting Information Figure S2c).

Lycopene Pathway Genes Can Be Regulated by tetO Variants. To determine whether tetO variants could modify the production of a compound in yeast, we chose lycopene production. Lycopene can be produced in yeast with the addition of three yeast codon-optimized genes from *E. herbicola*⁸ (Figure 4a), leading to cells with a yellow pigmentation that can be

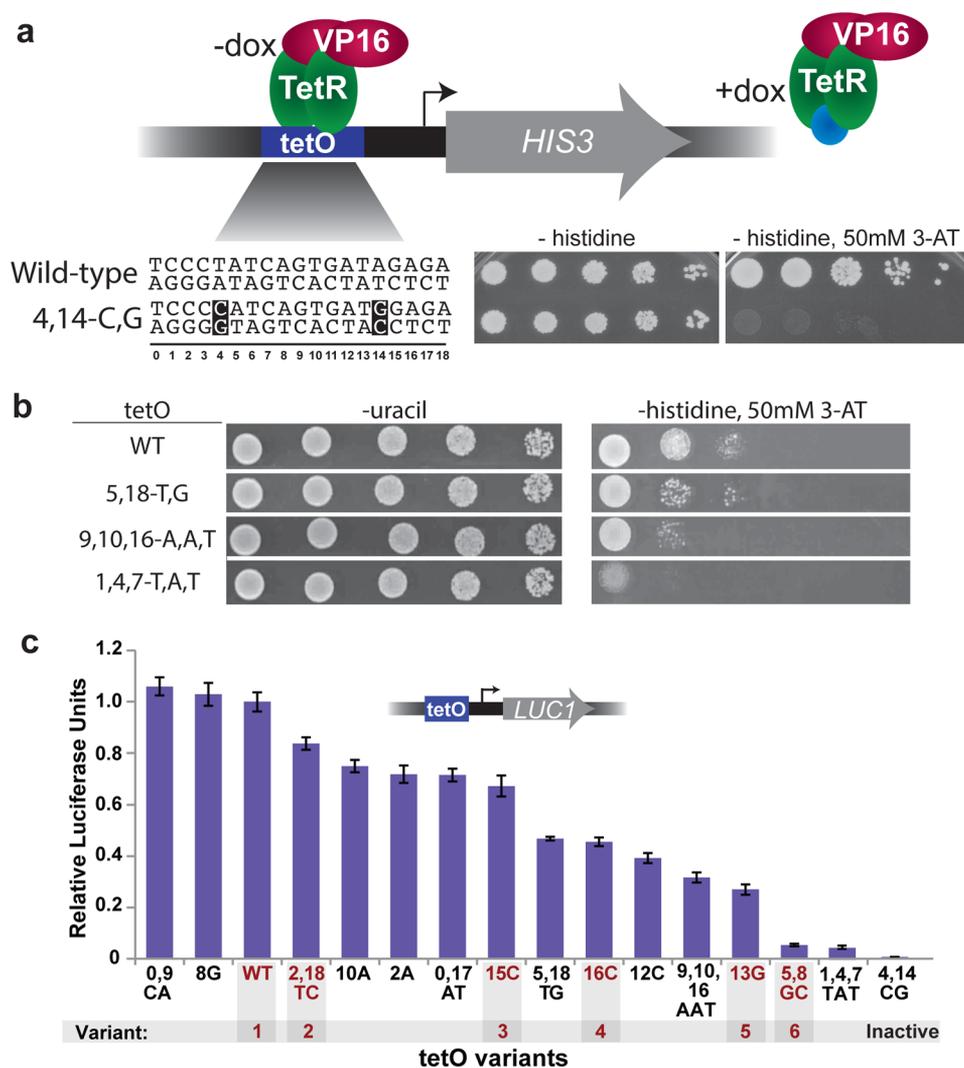


Figure 3. tetO variants result in differential Tet activator-driven expression. (a) Yeast are engineered to contain both the Tet activator (tetracycline repressor (TetR) fused to the VP16 transcription activation domain) and a *HIS3* gene driven by a tetO sequence. High level *HIS3* expression was dependent on a wild-type tetO site, as seen by the growth of yeast in the presence of 50 mM 3-amino-1,2,4-triazole (3-AT). The inactive 4,14-C,G doubly mutated variant of the tetO sequence did not support sufficient *HIS3* expression for growth on the 3-AT plate. 5-fold dilutions of normalized yeast cultures are shown. (b) Confirmation of three tetO variants, whose sequence changes are indicated, led to reduced growth on the 3-AT plate compared to that with the wild-type tetO sequence. Also 5-fold dilutions. (c) Mean (three biological replicates) \pm SE Tet activator-driven luciferase activity of 14 tetO variants identified by histidine selection were used to drive expression of the *LUC1* gene. Six tetO variants (in red) were chosen based on the relative expression that they generated and the presence of only one or two mutations from the wild-type (WT) sequence.

measured digitally from photographs or by OD₄₇₂ absorbance.¹⁷ We first placed the wild-type tetO sequence upstream of the *E. herbicola crtB*, *crtI*, and *crtE* genes involved in carotenoid biosynthesis (collectively referred to here as the *crt* genes) and cloned these genes into three separate yeast plasmids. Transformation of all three plasmids into yeast led to a high level of lycopene, as measured by both yellow pigment and absorbance (Figure 4, top row). This level of lycopene, $\sim 100 \mu\text{g}$ per gram dry weight, was similar to that observed previously in *S. cerevisiae*.¹⁸

To define the correlation between tetO variants and lycopene, we placed all three *crt* genes under the control of each of six tetO variants (the wild-type and five less active versions). Each combination of the three *crt* genes under the control of the same tetO variant was transformed into yeast. The concentration of lycopene, based on the color of the cells (a subset shown in Figure 4b, left) and extraction from the cells

(a subset shown in Figure 4b, right), correlated with predicted expression from the luciferase assay.

To identify the level of expression required from each *crt* gene for high lycopene production, we constructed three pools of plasmids corresponding to each of the *crtB*, *crtE*, and *crtI* genes under the control of each of the six tetO variants (Figure 4c). The plasmids were introduced iteratively into yeast by three sequential rounds of transformation, collection of transformants, and retransformation. We collected 672 triply transformed yeast colonies, spotted them on plates, and measured their color. On the basis of the intensity of their color, we divided the colonies into five bins (Figure 4d). Yeast colonies from within each color bin were pooled, and the three tetO-*crt* gene plasmids were extracted. After gene-specific amplification, we sequenced the tetO variants for each gene and calculated the proportion of each tetO variant in each bin (Figure 4e). Overall, there were differences in the number of transformation events for the six versions of each gene; for

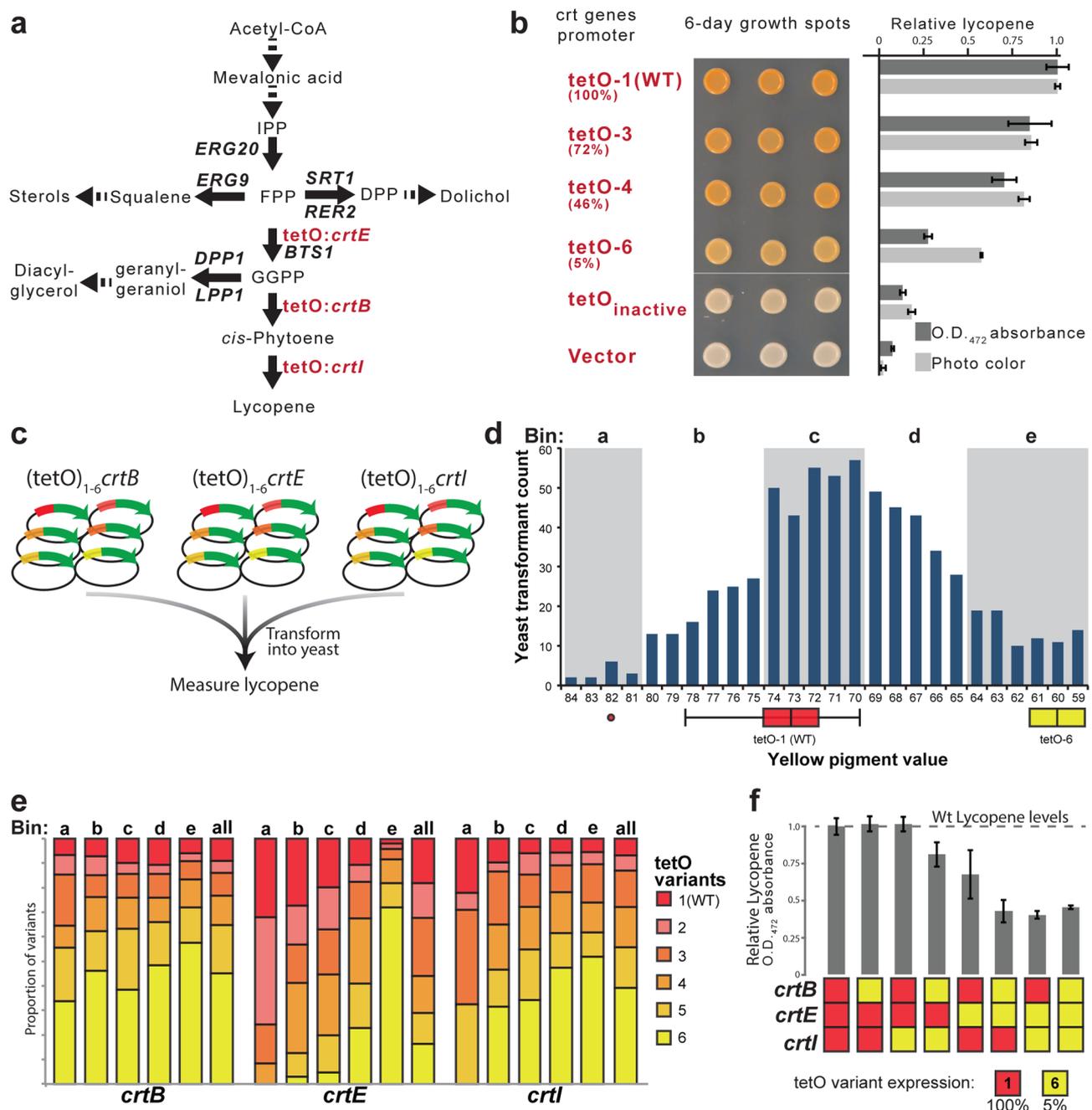


Figure 4. Use of the tetO variants to drive expression of genes for the production of lycopene in *S. cerevisiae*. (a) The isoprenoid biosynthesis pathway in *S. cerevisiae* plus three *crt* genes from *E. herbicola* whose expression is driven by tetO sequences result in the production of lycopene. (b) The three *crt* genes were transformed into *S. cerevisiae* under the control of the same tetO variant. The amount of lycopene produced was proportional to the potency of the tetO variant. Mean (three biological replicates) \pm SE of relative lycopene was measured by OD_{472} absorbance of ~ 10 million yeast cells extracted into dodecane or by measurement of yellow color. Spots in each row are replicates. (c) Pools of each *crt* gene under the control of the set of tetO variants were introduced iteratively into yeast, and 674 individual yeast colonies with all three *crt* genes were selected. There are 216 potential tetO–gene combinations. (d) After spotting, yeast colonies were measured for yellow pigment and ordered based on the yellow value. Five arbitrary bins were chosen based on yellow color. (e) Yeast in each color bin were combined, and deep sequencing of each of these bins resulted in unique distributions of tetO variants. The “all” proportions represent combined data from all five bins weighted based on the total number of yeast colonies. There is a strong bias toward high expression of *crtE* in yeast producing a high level of lycopene, based on the above average presence of tetO1-*crtE* in bins a and b. (f) Directed combinations of *crt* genes driven by either tetO-1 or tetO-6 confirmed the necessity for high expression of the *crtE* gene for yeast to produce a high level of lycopene. tetO-6 with either *crtI* or *crtB* in combination with tetO-1-*crtE* is sufficient to produce higher levels of lycopene (columns 2 and 3); however, low expression of both *crtI* and *crtB* leads to reduced lycopene production (column 4). Mean (three biological replicates) \pm SE extracted lycopene absorbance.

example, the tetO-6 *crtB* and *crtI* genes were present at higher levels than the other tetO variants in almost all of the bins. However, by comparing the relative levels of each tetO variant

in each bin, we observed that high expression of *crtE* correlated with high lycopene production and that *crtI* showed a weaker correlation compared to the overall proportions of these tetO

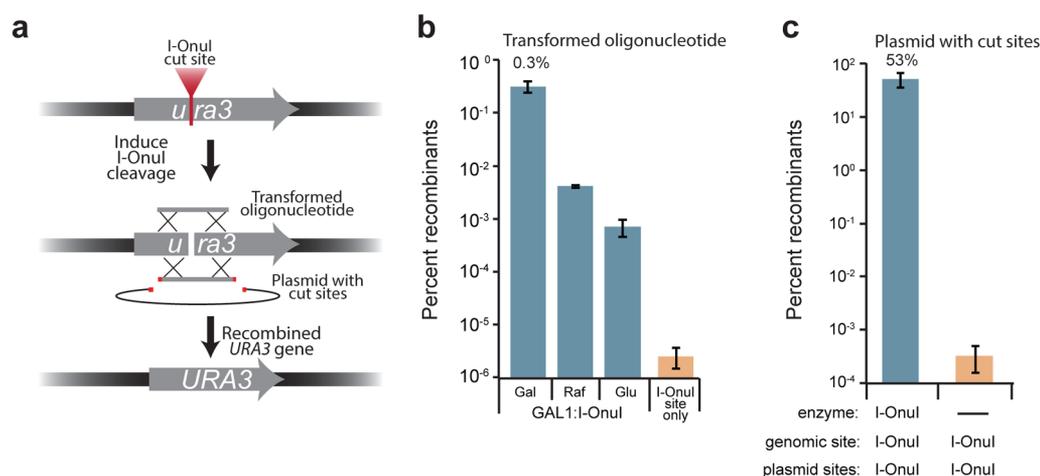


Figure 5. Homing endonuclease I-OnuI increases homologous recombination in *S. cerevisiae*. (a) Experimental design to compare homologous recombination rates. A site for either I-OnuI disrupts the *URA3* gene and becomes a double-strand break after galactose induction of I-OnuI, acting as a readout for successful template-mediated repair. Two assays were completed, one using transformed dsDNA as template and the other using template bordered with I-OnuI sites on a high copy number plasmid. (b) Mean ($n = 3$) \pm SE cells carrying I-OnuI expressed from the *GAL1* promoter were grown in galactose, raffinose, or glucose, and the number of Ura⁺ colonies was determined as a measure of recombination. Percentages represent the proportion of URA⁺ cells with the I-OnuI plasmid compared to cells with only the I-OnuI containing plasmid. (c) I-OnuI was expressed in cells carrying the I-OnuI site in *ura3* and in the plasmid carrying the oligonucleotide template for repair. The I-OnuI enzyme was induced for 6 h in galactose containing media. Mean ($n = 3$) \pm SE percentages represent the proportion of URA⁺ cells with the I-OnuI plasmid compared to that of cells with only the I-OnuI containing plasmid.

variants (Figure 4e, the “all” columns of *crtI* and *crtE*). Expression of *crtB*, however, was similarly distributed through the five bins. CrtE uses farnesyl diphosphate as its precursor to create geranylgeranyl diphosphate (Figure 4a). Farnesyl diphosphate is a key precursor molecule in endoplasmic reticulum membranes, lipid particles, mitochondrial complexes, and prenylated proteins¹⁹ and therefore may be limiting for lycopene synthesis. All colonies in bin a (11 total) had the tetO variants individually sequenced, which showed that the bin results reflected individual results (Supporting Information Figure S3a). None of these 11 colonies or colonies with any other combinations of tetO variants had any appreciable reduction in growth rate (Supporting Information Figure S3b).

To confirm the necessity for high expression of only *crtE*, we created combinations of either tetO-1 (WT) or low expression tetO-6 (5% expression compared to tetO-1) for all three *crt* genes and introduced them into *S. cerevisiae*. As expected, high expression of *crtE* was necessary to produce a high level of lycopene, whereas low expression of either *crtB* or *crtI* still allowed for this high level of lycopene (Figure 4f).

Enhancing Recombination in *S. cerevisiae*. To enhance the frequency of recombination events that would introduce tetO sites into the genome of *S. cerevisiae*, we introduced double-strand break sites near recombination sites (Figure 1). The homing endonuclease I-SceI has been used extensively both in *S. cerevisiae* and mammalian cells to induce double-strand breaks,²⁰ which, in yeast, boosts recombination rates by several orders of magnitude (\sim 4500-fold increase).²¹ More recently, the I-OnuI enzyme from *Ophiostoma novo-ulmi* led to a 9-fold increase in recombination over I-SceI in human cell culture.²² We tested the ability of I-OnuI to increase homologous recombination in *S. cerevisiae* by creating a nuclear-localized version of I-OnuI controlled by the galactose-inducible *GAL1* promoter on a low copy plasmid (pAG415 *GAL1*:I-OnuI). We tested our I-OnuI construct using an experimental design similar to that of other *S. cerevisiae* recombination assays.²¹ We first disrupted the *URA3* locus by

inserting an I-OnuI recognition site such that yeast could not grow in media lacking uracil. We then transformed these yeast with an oligonucleotide with 100 nucleotides of homology to the *URA3* locus and able to recombine and repair the *URA3* locus (Figure 5a). Recombination rates were determined by the relative number of cells able to grow on media lacking uracil compared to total viable cells. I-OnuI boosted recombination by 127 000-fold over cells without a double-strand break, about 30 times more effectively than that with the more commonly used I-SceI.²¹

While the rate of recombination with I-OnuI, 0.3%, approaches that of the *S. cerevisiae* strain created by DiCarlo et al.⁶ (0.7% recombination), we sought to further increase recombination by uncoupling the transformation and recombination steps. We bypassed the need for high-throughput transformation by first introducing a plasmid that harbors a template, flanked by I-OnuI digestion sites, with the potential to recombine with the *S. cerevisiae* genome. We used a *URA3* template bordered by I-OnuI cut sites (Figure 5a), introduced into yeast that carry a *URA3* gene disrupted by a homing endonuclease site. We induced the I-OnuI homing endonuclease for 6 h and plated the yeast on media lacking uracil to assay recombination. Induction of I-OnuI in an *S. cerevisiae* strain with an OnuI site disrupting *URA3* and a plasmid with a template capable of fixing *URA3* bordered by I-OnuI sites led to 53% of the cells recombining (Figure 5c). Without any induced cleavage, recombination occurred in only 0.0003% of cells.

Next, we applied both the tetO variants and the optimized recombination method to the production of lycopene. We integrated the three *crt* genes needed to produce lycopene into the *S. cerevisiae* genome, disrupting three pseudogenes on two chromosomes using template derived from the vector pRL-tetO-integration (see Methods). Each *crt* gene was regulated by the inactive tetO variant. Directly upstream of the inactive tetO variant we placed an I-OnuI cleavage site for efficient recombination (Figure 6a and Supporting Information Figure S4). As template, we generated a 6 \times -tetO plasmid harboring the six

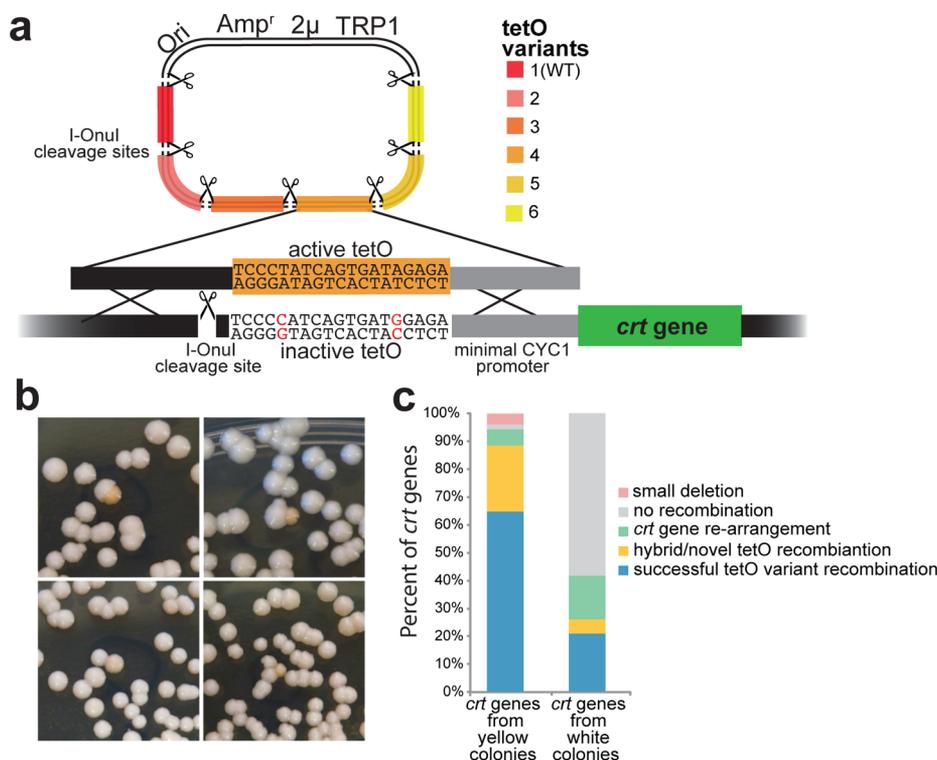


Figure 6. Proof of concept for the tetO toolkit. (a) tetO variant plasmid with six versions of the tetO sequence, each bordered by I-OnuI sites. Each tetO promoter can act as a template to recombine at any of the three integrated *crt* gene. (b) Plates showing colonies, some with yellow pigment indicating the production of lycopene. 0.73% of colonies total showed some amount of yellow pigment. (c) For the yellow colonies, 106 *crt* genes from 36 colonies could be characterized by sequence analysis, and for the white colonies, 78 *crt* genes from 28 colonies could be characterized. The majority of yellow colonies had an exact tetO recombination (64%), or hybrid recombination (24%), leading to a hybrid event of two tetOs. In white colonies, 21% of *crt* genes had an exact recombination event.

characterized tetO variants, separated by I-OnuI sites (pRS424-6 \times -tetO Figure 6a and Supporting Information Figure S5a,b). Each plasmid-borne tetO-template has 50-nucleotide arms with homology to the regions flanking the genomic tetO sites but does not contain the I-OnuI site. Thus, homologous recombination events should be stable and not further targeted by I-OnuI (Figure 6a). Altogether, this *S. cerevisiae* strain contained the integrated *crt* genes, *GAL1* promoter-driven I-OnuI, the Tet activator, and the tetO variant-template plasmid. After outgrowth and plating, colonies were visually inspected for yellow pigment (Figure 6b). Sixty-seven colonies showed yellow color out of 9097, a 0.73% rate of lycopene-producing yeast.

We characterized the integrated *crt* gene sites in 36 of the yellow colonies as well as 28 white colonies that did not express a high level of lycopene. The vast majority of yellow colonies had a tetO site recombination event at each of the three genes (93 of 106 *crt* genes, or 88%, including recombination events that led to hybrid tetO sites), whereas recombination occurred at 21 of 78 *crt* genes (27%) in the non-lycopene-producing cells, including events that led to hybrid tetO sites (Figure 6c and Supporting Information Tables S1 and S2). Of the tetO recombination events in yellow colonies, 68 of 106 (64%) were an exact recombination to one of the tetO variants carried on the tetO-template plasmid, whereas 25 of 106 (24%) created a hybrid tetO site (Figure 6c and Supporting Information Table S1). Among the exact tetO recombination events, variants tetO-2, tetO-4, and tetO-6 recombined at higher rates (15, 20, and 23%, respectively, of the *crt* genes in yellow colonies), whereas variants tetO-1, tetO-3, and tetO-5

recombined at lower rates (1, 1, and 5%, respectively, of *crt* genes in yellow colonies). Differences in rates of recombination dependent on different versions of gene sequences have also been seen with other recombination systems.⁶ The high-expression tetO-1 (WT) variant was present in low abundance at *crt* genes in either yellow or white colonies. However, a tetO-1/14G hybrid, in which only half of the tetO site recombined to the wild-type version, was commonly found (10% of *crt* genes from yellow colonies), indicating that this hybrid was sufficient for high level Tet activator-driven expression. Of the 78 recombination events of *crt* genes in white colonies, 16 (21%) were an exact recombination of a tetO variant, whereas 5 (6.4%) resulted in a hybrid tetO site (Supporting Information Table S1). In addition to the tetO recombination, recombination between *crt* genes occurred (observed in 15 cases (15%) in white colonies and 6 cases (5.6%) in yellow colonies), presumably because of common sequence such as the *CYC1* terminator used for all three genes, which could act as a template for complete gene conversion. Some small deletions occurred as well (observed in 3.8% of yellow colonies), leading to loss of the I-OnuI site entirely.

DISCUSSION

In this work, we describe a yeast-based toolkit to make quantitative changes in the expression of multiple genes by highly efficient recombination at targeted genomic sites. First, we optimized the tetO–Tet activator system for *S. cerevisiae* by identifying a yeast promoter for the gene encoding the Tet activator that leads to high level expression of tetO-driven genes. Second, we identified tetO variants that, in conjunction

with the Tet activator, result in more than a 100-fold range of expression. Third, we greatly increased homologous recombination by the use of the homing endonuclease I-OnuI and the introduction of I-OnuI sites in both the genome and on a plasmid.

Each of the components of the toolkit has advantages over existing technologies. While several versions of the Tet-Off system are used in *S. cerevisiae*, most use mammalian-optimized expression based on CMV-driven expression of the Tet activator.^{16,23–25} By optimizing the expression of the Tet activator, we boosted tetO-driven expression more than 30-fold over that with a previous Tet-Off system (Figure 2). The tetO variants we identified yield differential expression levels, whereas other eukaryotic Tet activator-based systems use only the single wild-type site. Presumably, a similar degree of differential expression could be attained in a mammalian cell culture with tetO variants. In addition to the usefulness of these variants in a genome engineering context, they could be used as stand-alone elements to direct expression of exogenous genes. I-SceI has been the most commonly used homing endonuclease to induce double-strand breaks in both yeast and mammalian cells. We found I-OnuI to be extremely efficient for inducing recombination by double-strand break repair, leading to a ~30-fold increase over previously published I-SceI rates (Figure 5B).²¹

We applied the tetO variants and I-OnuI endonuclease and targeting site to the three genes required for lycopene production in yeast. Recombination occurred at a high rate, with 88% of the yellow colonies having undergone a recombination at each site. While some alternative recombination events occurred among the *crt* genes, many were likely gene conversions due to identical sequences (in the terminator and promoter) in all three genes. Future applications of this technology would not include such repeated elements. While we observed high recombination rates overall, there were unaccountable differences between the rates of recombination of each of the tetO variants (Tables S1 and S2). Possibly, this difference is due to the construction and order of the tetO variants on the 6X-tetO plasmid, as tetO-6 is at the terminus of the set of variant elements (Figure 6a and Supporting Information Figure S5). Alternatively, there could be a selection bias for tetO variants that have low expression, although we saw no evidence of growth defects associated with high expression (Supporting Information Figure S3). Differential recombination rates could be a limitation of this approach generally or could be specific to the production of lycopene with this version of the reagents. Potentially reordering the elements on the 6X-tetO plasmid, or using a mixture of plasmids with differing element placement, could eliminate the tetO-6 recombination bias. A large proportion (24%) of yellow colonies had partial recombination of the tetO site. These partial events must result in tetO elements at least functional enough to confer enough Tet activator-driven expression for lycopene.

While other methods to induce multiplex changes in gene expression have been described, this work allows for extremely high rates of recombination. In our experiments, we induced I-OnuI in galactose-containing media for 6 h, and by increasing the induction time, we might increase the recombination rate even further. Recently, genome editing using bacterial clustered regularly interspaced palindromic repeats (CRISPR-Cas9) has yielded high recombination rates in *S. cerevisiae*.²⁶ Future iterations of this toolkit could include the use of the CRISPR-Cas9 system to induce double-strand breaks at existing genomic

sequences, without the need to introduce I-OnuI sites. Other transformation-based methods such as reiterative recombination⁸ lead to multiple recombination events; however, each round of recombination requires an additional round of transformation, making its application to several genes at once limiting.

The tetO toolkit could have numerous applications in yeast. For example, targeting the native isoprenoid pathway would be useful for enhancing production of several yeast-produced small molecules, including artemisinic acid (precursor for artemisinin), taxol, beta carotene, astaxanthin, protopanaxatriol, and several steroids.²⁷ Alternatively, for the production of medically important proteins like the one used in the HPV vaccine,²⁸ expression of the enzymes involved in folding, degradation, glycosylation, or secretion could be targeted.²⁹ Overall, this toolkit can deliver expression elements of variable potency to a set of genes in yeast at a recombination rate of about 20% per locus, making it highly versatile for genome engineering.

METHODS

Strains and Media. Yeast experiments used the BY4741 strain with a *trp1* knockout created by Giaever et al.³⁰ Tet activator expression analysis was performed by ligating the Tet activator gene into the Funk et al.¹³ vectors with *URA3* marker (Supporting Information Table S4). Cells were grown at 30 °C in synthetic complete media lacking uracil and supplemented with 2% glucose. Plasmid-based tetO-*crt* gene combinations were cloned into the pRS series³¹ of yeast vectors with *LEU2*, *TRP1*, or *HIS3* nutrient marker. Eukaryotic codon-optimized I-OnuI was amplified from pETCON-I-OnuI³² and ligated into pAG415Gal1³³ with the addition of an SV40 nuclear localization signal. GAL1:I-SceI was digested from pGSKU²¹ and ligated into pRS425.³¹

Transformation Protocol. Yeast cells were grown 24 h in minimal media with 2% raffinose. Cells were back-diluted to OD₆₀₀ of 0.2 in 4% raffinose; 2% raffinose, 2% galactose; or 2% raffinose, 2% glucose. Cells were grown for 4 h, resuspended in an equal volume of 0.15 M lithium acetate, and incubated at room temperature for 1 h. Yeast were then combined with 5 μL of denatured carrier DNA, 1 μg of linear or plasmid DNA, and 500 μL of 0.15 M lithium acetate, 40% polyethylene glycol 4000, 1× TE and incubated for 1 h. After 1 h, DMSO was added to a concentration of 10%, and incubation was continued at 42 °C for 15 min. Cells were resuspended in water and plated on minimal selective plates. To assess transformation efficiency, 1:10000 and 1:100000 dilutions were plated on non-selective media.

Oligonucleotides and DNA Sequencing. Oligonucleotides were obtained from Integrated DNA Technologies with standard desalting purification. Sanger sequencing and analysis was performed as previously described.³⁴ Deep sequencing of plasmid DNA was performed on an Illumina Miseq by purifying DNA using the ZymoPrep yeast plasmid prep II (Zymo Research) and PCR amplification for 12 cycles.

tetO Regulation of *HIS3*. The endogenous promoter of *HIS3* was replaced from –1 to –122 bp upstream from the start codon with a single tetO site derived from the previously published construct pCM188.¹⁶ The tetO site was placed directly upstream of a 140 bp minimal *CYC1* promoter. Using primers with complementary sequence, we introduced *HIS3* under the control of the tetO promoter into the BY4741 *S. cerevisiae* strain containing a partial *HIS3* deletion.

URA3 Allelic Replacement Assays. Efficiency of replacement was characterized by introducing a homing endonuclease recognition site (either I-SceI or I-OnuI, Supporting Information Table S3) within the gene body of *URA3* at coding position 334, disrupting gene function. For the oligonucleotide-based assay, we used 1 μg of double-stranded oligonucleotide (Supporting Information Table S3) capable of fixing *URA3* following transformation. Replacement of the functional portion of *URA3* was scored by the ability of yeast to grow on media lacking uracil. To assess relative recombination rates, we compared the number of the *URA3*-containing yeast colonies to the total number of yeast colonies that contained the plasmid encoding the homing endonuclease. For the plasmid-based assay, we first introduced two plasmids, one with the template containing the *URA3* sequence and one encoding the endonuclease. Relative recombination was the number of *URA3*-containing yeast colonies divided by the total number that contained the two plasmids.

RNA Extraction, cDNA Synthesis, and Quantitative PCR. One milliliter of yeast was grown to mid log phase and centrifuged at 3500g for 5 min. The supernatant was removed, and cell pellets were resuspended in 400 μL of lysis buffer (10 mM Tris-Cl, pH 7.5, 10 mM EDTA, 0.5% SDS), and 400 μL of acid phenol was added. Samples were incubated at 65 $^{\circ}\text{C}$ for 1 h with shaking, placed on ice for 10 min, and centrifuged at 14 000g for 10 min at 4 $^{\circ}\text{C}$. The aqueous phase was removed, combined with 400 μL of chloroform, and vortexed. Samples were centrifuged at 14 000g for 10 min again, and the aqueous phase was combined with 0.1 volumes of 3 M sodium acetate and 2.5 volumes of ethanol, incubated on ice, and centrifuged for 10 min at 14 000g. Pellets were washed once with 70% ethanol, dried, and resuspended in nuclease-free water. The extracted RNA was quantified using the ND-1000 spectrophotometer (NanoDrop Technologies).

Oligonucleotides for real-time PCR were designed using the NCBI primer-blast (Supporting Information Table S3). Housekeeping gene primers (*TAF10*) were taken from Teste et al.³⁵ Approximately 50 ng of RNA was treated with turbo DNase (Life Technologies) following the manufacturer's specifications. RNA was reverse-transcribed into cDNA in a 20 μL reaction using the Superscript III kit (Life Technologies). Using a 1:50 dilution of cDNA, three biological replicates were amplified using 1 unit Phusion polymerase (New England Biolabs) with SYBR green and 0.5 μM of each primer on the ABI Light Cycler. PCR conditions included a single hold at 98 $^{\circ}\text{C}$ for 3 min followed by 40 cycles of 98 $^{\circ}\text{C}$ for 15 s, 60 $^{\circ}\text{C}$ for 15 s, and 72 $^{\circ}\text{C}$ for 30 s. After completion of the PCR, a melting curve was performed to verify that no contamination was present. Relative expression was normalized to *TAF10* expression using the $\Delta\Delta C_t$ method.

Luciferase Assay. Cultures were grown overnight at 30 $^{\circ}\text{C}$ in selective minimal media to mid log phase, and the optical densities at 660 nm were measured. One-hundred microliters of cells was mixed with 100 μL of 1 mM D-luciferin in 0.1 M sodium citrate, pH 3, in a black plate, and luminescence was measured on a PerkinElmer Victor 3 V plate reader after a 2 min incubation at room temp. Luminescence values were divided by the OD to determine a relative measurement. Values presented in figures are mean values ($n = 3$) \pm standard deviation.

Lycopene Measurement. Cells were grown for 16 h until saturated. One milliliter of cells was pelleted, and media was removed. A 200 μL volume of 425–600 μm diameter glass

beads and 300 μL of dodecane were added. Cells were shaken using a mini beadbeater (Biospec Products) for 3 min and then pelleted by centrifugation. Two-hundred microliters of dodecane was used to measure OD₄₇₂ and samples were normalized to the cell density at OD₆₆₀. Absorbance was normalized to pure dodecane for OD₄₇₂ measurements, and relative lycopene concentrations were set to a wild-type tetO level of 1.0. Standard error of the mean was calculated by taking the standard deviation divided by the square root of the number of biological replicates.³⁶ Color was measured by first spotting each strain on plates (Figure 4b) and then using Adobe Photoshop's eye dropper tool to measure the yellow channel level from CMYK color from a 51 pixel diameter circle at the center of each spotted colony (approximately 20% of total area). No in-depth colorimetric measurement was done for Figure 6; in this case, colonies with yellow color were identified by eye and grown in liquid culture, and DNA was extracted as previously described.

Plasmid-Based tetO Variant Assay. TetO-1 through tetO-6 were introduced upstream of each gene *crtB*, *crtE*, and *crtI* with each encompassed on a 75-nucleotide sequence. Codon-optimized *E. herbicola crtE*, *crtB*, and *crtI* genes⁸ were introduced into *TRP1*, *HIS3*, and *LEU2* nutrient marker-containing plasmids, respectively. Three rounds of yeast transformation led to 672 transformants that were further characterized by culturing in 96-well plates in 200 μL of selective minimal media overnight at 30 $^{\circ}\text{C}$. Then, 10 μL was spotted on minimal media plates and grown at 30 $^{\circ}\text{C}$ for 4 days. Pictures of the plates were taken, and photocolometric measurements were made using Adobe Photoshop. Variants were separated into five bins based on the yellow channel values of the Photoshop measurements. Individual yeast strains in each bin were combined and grown at 30 $^{\circ}\text{C}$ overnight, and plasmids were collected using the zymoprep yeast miniprep kit II (Zymogenetics). The "all" column in Figure 4e refers to a weighted proportion based on the number of yeast colonies from each of the five bins.

Growth Rate Measurements. Yeast cultures were grown overnight at 30 $^{\circ}\text{C}$ until saturated. In 96-well plates, cultures were diluted 1:20 in a 200 μL volume of minimal selective media. The plates were continuously shaken at 30 $^{\circ}\text{C}$ in selective media with a synergy H1 hybrid reader (Biotek). Mean ($n = 3$) maximum doubling rate was determined by measuring the largest slope of OD₆₆₀ measurements over a 2 h period \pm standard deviation.

crt Gene Integration. An integration vector (pRL-tetO-integration) was created by generating a fragment that includes an XhoI site, linker sequence, an I-OnuI site, a 4,14C-G (inactive) tetO variant site, a *URA3* gene, another I-OnuI site, another 4,14C-G tetO variant site, other linker sequence, a *CYC1* promoter, a multiple cloning site (MCS), a *CYC1* terminator, and another XhoI site (see map and sequence in Supporting Information Figure S4). The fragment was cloned into the compatible SalI site within the MCS of pRS413. The *crt* genes were cloned into the BamHI and ClaI sites within the MCS of pRL-tetO-integration via Gibson cloning as previously described.³⁷ To generate a final fragment for transformation and integration, primers annealing to the T7 and M13 reverse sequences of pRS413 were created with 50 base extension tails homologous to the insertion site within the yeast genome. The PCR fragment was transformed into BY4741 $\Delta trp1$, and colonies were selected on plates lacking uracil. Resulting colonies were sequence-verified and then grown in nonselective

liquid media overnight followed by plating on 5-fluoroorotic acid (5-FOA). Clones in which an homologous recombination event had occurred between the two tandem 64 bp I-OnuI/4,14-C,G TetO/flanking sequence cassettes should lead to the pop-out and loss of the *URA3* gene (leaving behind one copy each of the I-OnuI recognition site and 4,14-C,G TetO site upstream of the *CYC1* promoter), thus allowing the cell to grow on 5-FOA. The *URA3* marker was then available for the next *crt* gene integration, and the process was repeated until all three *crt* genes were inserted into the genome. *crtB* was inserted antisense into the pseudogene YDL242W, *crtE* was inserted antisense into the pseudogene YBR032W, and *crtI* was inserted antisense into the pseudogene YBR144C.

tetO Toolkit Implementation. After integration of pRL-tetO-integration segments, pCYC1:Tet activator (*URA3* marker), pRS424-6X-tetO (*TRP1* marker), and pAG415 GAL1:I-OnuI (*LEU2* marker) were introduced by iterative transformation as previously described. Eighty colonies were scraped and grown overnight in galactose and replated on YEPD, SC complete, and SC minus uracil plates. Lycopene-producing colonies were identified on YEPD plates and were transferred to SC complete and SC minus uracil plates for further analysis. Further description of pRS424-6X-tetO and pRL-tetO-integration can be found in Supporting Information Figures S4 and S5.

■ ASSOCIATED CONTENT

■ Supporting Information

Figure S1: Effects on tetO:LUC1 expression and doubling time of the Tet activator driven by several yeast promoters. Figure S2: tetO variants result in differing expression levels, which are dependent on the Tet activator. Figure S3: Individual sequencing of *crtE*, *crtB*, and *crtI* plasmid-containing strains match high-throughput sequencing, and higher production of lycopene has no effect on yeast growth. Figure S4: Map of pRL-tetO-integration vector. Figure S5: Map and sequence of pRS424-6X-tetO. Table S1: Summarized results of tetO recombination of *crt* genes. Table S2: Individual colony recombination of yeast colonies producing lycopene and those not producing lycopene. Table S3: List of primers used in this study. Table S4: List of plasmids used in this study. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Virginia Cornish for plasmids containing codon-optimized lycopene genes, Andrew Scharenberg, Kyle Jacoby, and Abigail Lambert for the I-OnuI containing plasmid, and Douglas Fowler for comments on the manuscript. This work was supported by grant P41 GM103533 from the National Institute of General Medical Sciences of the National Institutes of Health. S.F. is an investigator of the Howard Hughes Medical Institute.

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