Synthetic and Systems Biotechnology 9 (2024) 820–827

Contents lists available at ScienceDirect

Synthetic and Systems Biotechnology

journal homepage: www.keaipublishing.com/en/journals/synthetic-and-systems-biotechnology

Original Research Article

Cyanamide-inducible expression of homing nuclease $^{I-}$*Sce*I for selectable marker removal and promoter characterisation in *Saccharomyces cerevisiae*

Liam McDonnell [a,b,1], Samuel Evans [a,b,1], Zeyu Lu [a,b,c], Mitch Suchoronczak [a], Jonah Leighton [d], Eugene Ordeniza [d], Blake Ritchie [d], Nik Valado [d], Niamh Walsh [d], James Antoney [a,b], Chengqiang Wang [e], Carlos Horacio Luna-Flores [a], Colin Scott [f], Robert Speight [a,b,g], Claudia E. Vickers [a,b], Bingyin Peng [a,b,c,*]

[a] *Centre of Agriculture and the Bioeconomy, School of Biology and Environmental Science, Faculty of Science, Queensland University of Technology, Brisbane, QLD, 4000, Australia*
[b] *ARC Centre of Excellence in Synthetic Biology, Australia*
[c] *Australian Institute for Bioengineering and Nanotechnology (AIBN), The University of Queensland, Brisbane, QLD, 4072, Australia*
[d] *School of Biology and Environmental Science, Faculty of Science, Queensland University of Technology, Brisbane, QLD, 4000, Australia*
[e] *College of Life Sciences, Shandong Agricultural University, Taian, Shandong Province, 271018, People's Republic of China*
[f] *CSIRO Environment, Black Mountain Science and Innovation Park, Canberra, ACT, 2601, Australia*
[g] *Advanced Engineering Biology Future Science Platform, Commonwealth Scientific and Industrial Research Organisation (CSIRO), Black Mountain, ACT, 2601, Australia*

ARTICLE INFO

Keywords:
Metabolic engineering
Genetic circuit
Genome engineering
Synthetic biology
Yeast engineering
Promoter engineering

ABSTRACT

In synthetic biology, microbial chassis including yeast *Saccharomyces cerevisiae* are iteratively engineered with increasing complexity and scale. Wet-lab genetic engineering tools are developed and optimised to facilitate strain construction but are often incompatible with each other due to shared regulatory elements, such as the galactose-inducible (*GAL*) promoter in *S. cerevisiae*. Here, we prototyped the cyanamide-induced $^{I-}$*Sce*I expression, which triggered double-strand DNA breaks (DSBs) for selectable marker removal. We further combined cyanamide-induced $^{I-}$*Sce*I-mediated DSB and maltose-induced MazF-mediated negative selection for plasmid-free *in situ* promoter substitution, which simplified the molecular cloning procedure for promoter characterisation. We then characterised three tetracycline-inducible promoters showing differential strength, a non-leaky β-estradiol-inducible promoter, cyanamide-inducible *DDI2* promoter, bidirectional *MAL32/MAL31* promoters, and five pairs of bidirectional *GAL1/GAL10* promoters. Overall, alternative regulatory controls for genome engineering tools can be developed to facilitate genomic engineering for synthetic biology and metabolic engineering applications.

1. Introduction

In synthetic biology and metabolic engineering, yeasts have been intensively engineered to produce and improve production of certain fuels, chemicals, and other bioproducts [1–4]. It often requires genetic modification on multiple genes and at multiple genomic loci to generate ideal genotypes and phenotypes [5,6]. Such modification capacity is rapidly evolving as the result of developing various engineering tools, including computational design/learn tools, laboratory build/test tools, and high throughput procedures assisted by robotic automation [7–11]. Wet-lab strain engineering is essentially important in this process but often labour-intensive and resource-consuming [12]. To accelerate the process, strain engineering tools and strategies can be further explored.

Genomic integration of transgenes in the common yeast *Saccharomyces cerevisiae* was first reported in the 1970s [13]. Following this, genomic engineering methodologies and strategies, including plasmid vectors [14,15], positive/negative selectable markers [16–18], multi-gene introduction [19–21], and large-scale genome synthesis [22,

Peer review under responsibility of KeAi Communications Co., Ltd.
* Corresponding author.
E-mail address: bingyin.peng@qut.edu.au (B. Peng).
[1] Joint first author.

https://doi.org/10.1016/j.synbio.2024.06.009
Received 7 April 2024; Received in revised form 27 June 2024; Accepted 27 June 2024
Available online 28 June 2024

23], have been continuously developed and improved. In rational yeast engineering, gene knock-out and gene knock-in are essential to deliver identical genotypes according to the research design (or hypotheses). Inherit advantages in *S. cerevisiae* include its highly efficient homologous recombination, whereas other non-conventional yeasts, like *Komagataella phaffii* (previously known as *Pichia pastoris*), may be engineered to reach the comparable efficiency as well [24,25]. Through homologous recombination, endogenous genes can be deleted through a knock-out strategy and heterologous genes can be integrated through a knock-in strategy. To facilitate knock-out/knock-in engineering processes, molecular biology tools are deployed, i.e., tyrosine recombinase-mediated site-specific recombination (like Cre and Flp) [26], CRISPR-associated nuclease-mediated genome double-strand breaking (like Cas9 and Cpf1) [27], meganuclease-mediated genome double-strand breaking (like the homing nuclease I-*Sce*I) [28], and serine recombinase-mediated site-specific recombination (like Bxb1 and PhiC31) [29]. These tools may be further modified for alternative purposes. For example, Cre-loxP recombination is used for SCRaMbLE [30], synthetic chromosome rearrangement and modification by loxP-mediated evolution. CRISPR tools are used for CRISPRi (CRISPR interference) [31] and CRISPRa (CRISPR activation) [32]. Ideally, all these tools can be compatible with each other to realise their functions in a single host, allowing the engineering and integration of complexed biological systems.

Some of these molecular biology tools are not compatible with each other for parallel applications in the host. For example, galactose-inducible promoters are used to control the induction of Cre-LoxP-mediated SCraMbLE [33], Cre-LoxP-mediated marker removal [34], $^{I-}$*Sce*I-mediated marker excision [28], $^{I-}$*Sce*I-mediated on-genome assembly of multiple genes [19], and mostly the induction of heterologous metabolic pathways in metabolic engineering [35–37]. To solve the compatibility problem, orthogonal genetic regulatory systems can be used to control each of these engineering machineries. In *S. cerevisiae*, both native and synthetic regulatory systems have been widely investigated in synthetic biology and yeast engineering studies [3,38,39]. The cyanamide-inducible promoter (*DDI2*) and maltose-inducible promoter (*MAL32*) are the options of native systems, and do not require the introduction of additional synthetic regulatory factors [40–42]. Therefore, we aimed to explore their usage for the orthogonal control on genome engineering tools.

In this study, we developed a cyanamide-inducible $^{I-}$*Sce*I system for selectable marker removal. Incorporating the maltose-inducible expression of the bacterial toxin gene MazF [18], we further developed an *in-situ* promoter substitution system to facilitate promoter characterisation. Using this tool, we characterised five categories of inducible promoters.

2. Materials and methods

2.1. Plasmid and strain construction

Yeast strains used in this study are listed in Supplementary Table 1. Plasmids used in this study are listed in Supplementary Table 2. Primers used in yeast colony PCR are listed in Supplementary Table 3. The *CYC1core[4×Z268 + 1×LmrO]* promoter sequence is listed in Supplementary Information file.

2.2. Yeast cultivation and transformation

Yeast extract Peptone media (YP media; 10 g L^{-1} yeast extract, and 20 g L^{-1} peptone), supplemented with 20 g L^{-1} glucose (YP-glucose media), were used in general strain maintenance and development. Antibiotic G418 sulphate (300 μg mL^{-1}) or hygromycin B (100 μg mL^{-1}) was used in the selection of the strain harbouring the *KanMX4* or *HphMX* selectable marker [34]. Yeast nitrogen base media (YNB media; 6.9 g L^{-1} yeast nitrogen base without ammonia sulphate and ammino acids, pH 6.0), were used to grow the strain harbouring the *URA3* selectable marker in yeast transformation and characterisation. When maltose and/or galactose were used as the carbon source(s), each of them was supplemented at the concentration of 20 g L^{-1}. For characterisation of genetic induction, 2 mM cyanamide (diluted from 4 M stock in H_2O), 125 μM tetracycline (diluted from 125 mM stock in dimethylsulfoxide: ethanol 1:1 mixture; stored at −80 °C), or 1 μM β-estrodiol (diluted from 1 mM stock in dimethylsulfoxide) was used. Agar (20 g L^{-1}) was added to prepare the solid media.

S. cerevisiae transformation was performed using the LiAc/ssDNA/PEG method with modifications [43]. Yeast cells from the recovery plate were inoculated into YP-glucose media at OD_{600} = 0.001 for routine transformation or 0.0004 for the transformation with 2-h cyanamide induction and grown overnight. The LiAc/SScarrierDNA/PEG method [43] was followed to prepare the transformation mixture with heat-shock treatment. For antibiotic selection, cells collected from transformation mixture were precultured for at least 3 h in YP-glucose media and then spread onto the selection agar plates for further cultivation. For the yeast promoter knock-in with the negative selection through maltose-induced MazF toxin expression, cells were precultured in 1 mL YP-glucose media and incubated at room temperature overnight, and 10 μL of overnight culture was spread onto each YP-maltose agar plate supplemented with the required inducer like galactose, tetracycline, cyanamide, or β-estrodiol. After 48-h incubation at 30 °C, the plates were imaged using a BioRad ChemiDoc Imaging System.

For yeast promoter characterisation, yeast cells were pre-cultured in 200 μL YNB-glucose media in 5 ml sterile falcon tubes for ~24 h to the stationary phase. The cultures were grown in a 200 rpm 30 °C incubator. For characterisation in YNB-glucose media, the precultures were diluted by 1000 times in 200 μL YNB-glucose media with or without inducer supplement. For the characterisation in YNB-galactose media, the precultures were diluted by 100 times in 200 μL YNB-galactose media. For the characterisation in YP media, the precultures were diluted by 10,000 times in 200 μL fresh YP-glucose or YP-maltose media. The diluted cultures were grown overnight for fluorescence measurement through flow cytometer.

2.3. Yeast colony PCR

Yeast colony PCR was performed using the protocol we published previously [5]. Yeast cells were resuspended in 5 μL of yeast cell digestion solution, the 10:1 mixture of 1 × phosphate-buffered saline (PBS) buffer and Zymolyase-20 T (nacalai tesque, Japan) stock solution (1U per μL in 1 × PBS buffer containing 100 mM DTT and 50 % v/v glycerol). Yeast cells were digested at 37 °C for 30 min, denatured at 95 °C for 5 min, and diluted with 100 μL of water. The mixture (1 μL) was used as the template in 10 μL of PrimeSTAR GXL DNA Polymerase (Takara Bio Inc., Japan) reaction. The typical PCR thermal-cycle conditions are (1) 98 °C for 30 s, (98 °C for 15 s, 50 °C for 10 s, 68 °C for 5 min)*3, (98 °C for 15 s, 55 °C for 10 s, 68 °C for 5 min)*27, 68 °C for 5 min. The extension duration at 68 °C was adjusted according to the amplicon length.

2.4. Flow cytometry

A BD FACSCanto II flow cytometry (Firmware version 1.49) was used to measure yEGFP [44] and E2Crimson [45] fluorescence in single cells. Yeast cells in overnight cultures were used directly for fluorescence analysis. yEGFP fluorescence was monitored at the FITC-A channel with a 488 nm laser. E2Crimson fluorescence was monitored at the APC-A channel with a 633 nm laser. Mean values of FSC-A (PMT voltage = 200; gating threshold = 3000), SSC-A (PMT voltage = 200), FITC-A (PMT voltage = 450), and APC-A (PMT voltage = 535) for all detected events were extracted using BD FACSDiva Software (version 8.0.1; CST Version 3.0.1; PLA version 2.0). yEGFP and E2Crimson fluorescence levels were expressed as the percentage of the average background

autofluorescence from the exponential-phase cells of FP-negative reference strain CEN.PK113-7D [46].

3. Results

3.1. Cyanamide-inducible expression of $^{I-}$SceI for selectable marker removal

In *S. cerevisiae*, $^{I-}$*Sce*I expression causes the double-strand break (DSB) at the 18 bp $^{I-}$*Sce*I recognition site (5′-AGTTACGCTAGGGATAA-CAGGGTAATATAG-3′) [19,28]. DSBs trigger DNA repair, prevalently through homologous recombination in *S. cerevisiae* when homologous sequences surround $^{I-}$*Sce*I recognition site. This mechanism was applied to facilitate selectable marker removal and the assembly of multiple genes into genome [19,28]. Previously, galactose-inducible *GAL1* promoter was used to control $^{I-}$*Sce*I expression [19,28]. However, *GAL* promoters are broadly used to control the expression of heterologous genes in metabolic engineering, i.e., the genes in terpene synthetic pathways [5,14,47]. Induction of *GAL* promoters-controlled terpene pathway genes may cause metabolic burden, challenging the strain development and therefore not compatible with *GAL* promoter-mediated induction of $^{I-}$*Sce*I expression. We aimed to use an alternative regulatory promoter to control $^{I-}$*Sce*I expression to facilitate strain engineering. The *DDI2* promoter was considered because of its binary 'OFF-to-ON' induction in the presence of cyanamide [40,41].

We constructed a two-vector system to introduce the $^{I-}$*Sce*I expression cassette under the control of the *DDI2* promoter (Fig. 1A: pITE-SceIcmd & pITEdv1). These two plasmids were co-transformed into CEN.PK113-32D [46] genome with the replacement of the *HO* gene, the gene required for mating-type switch in natural *S. cerevisiae* isolates (Fig. 1A: strain oQIE0 with genotypes confirmed by yeast colony PCR). As the results, the 174-bp repeated sequences were at the both ends of the fragment $^{I-}$SceI_Site-*HphMX* (the hygromycin resistant selectable marker)-$^{I-}$*Sce*I_site. We grew oQIE0 in YPD broth supplemented with 2 mM cyanamide from a low OD_{600} ~ 0.001 for 20 h and then isolated the single colonies by spreading the diluted culture on a YPD agar plate supplemented with 2 mM cyanamide. We tested 24 colonies through yeast colony PCR, and the Hph gene was successfully removed in ~75 % of the colonies, shown by the loss of the amplification of PCR fragment #3 (Fig. 1A: generating strain oQIE1).

To further investigate the applications of the marker-recycling mechanism, we re-constructed the background platform strain for improved synthesis of heterologous terpenoids. Firstly, we transformed the construct that encoded the genetic circuit for tetracycline-mediated repression of *GAL* promoters (Fig. 1B: pIRTetR-GAL80) [38]. This construct is targeted to replace the promoter of the *GAL* repressor gene *GAL80* with the *TEF1* promoter inserted with 4 TetO elements, the G418 selectable marker *KanMX4*, and the expression cassette of an artificial transcription factor TetR-Tup1p. In this construct, we have previously cloned two 40-bp repeated sequences at the both ends of *KanMX4*-$^{I-}$*SceI*_site. Here, the 18-mer $^{I-}$*SceI*_site (5′-TAGGGATAACAGGGTAAT-3′) was used. This construct was transformed into strain oQIE1 to generate strain oQIE2 (Fig. 1B: with genotype confirmed by yeast colony PCR). After cyanamide-mediated induction of $^{I-}$*Sce*I, 27 colonies were characterised through yeast colony PCR. None of these colonies showed the successful removal of the *KanMX4* marker. Instead, each colony likely contained the mixture of two genotypes: *KanMX4* unremoved (Fig. 1B: PCR fragment #5) and *KanMX* removed (Fig. 1B: PCR fragment #7). We chose first four colonies (replicated on the plate without G418 supplemented) to perform the second round of cyanamide induction and tested 27 colonies for the G418 resistance. Five colonies had the *KanMX* marker removed (Fig. 1B: strain oQIE3). This case indicates that 40-bp repeats and a single 18-mer *SceI*_site do not work efficiently for marker removal.

We further aimed to transform two plasmids pIMVAd39T (Addgene #98303) and pIMVAu1 (Addgene #98301) to overexpress the genes from the mevalonate pathway [47]. In these two plasmids, autotrophic selectable markers were used for yeast transformation, but were not available in oQIE3. We further used the *KanMX4* marker containing two $^{I-}$*SceI* sites and the 80-mer AB repeats (EuroScarf#P20769: pDS1) [28], to investigate the efficiency of cyanamide-induced marker removal. We replaced the selectable marker in pIMVAd39T and pIMVAu1 with *AB*-$^{I-}$*SceI*-*KanMX4*-$^{I-}$*SceI*-*AB* fragment, and sequentially transformed them into oQIE3 with cyanamide-induced marker removal (Fig. 1C). The success rates for selectable marker removal were ~37 % and 33 %. These are lower than the efficiency (>50 %) reported previously when galactose-inducible promoter was used for $^{I-}$*SceI* induction [47].

3.2. In situ gene replacement design for plasmid-free cloning of heterologous promoters into yeast genome

The fundamental principles in metabolic engineering and synthetic biology rely on the expression of heterologous genes under the control of different promoters [48]. The promoters can be either constitutive or regulatory and show a range of transcription strengths [49]. Nevertheless, it is important to characterise these features for genetic design in metabolic engineering and synthetic biology. In *S. cerevisiae*, promoters can be characterised using an episomal plasmid system or a genome-integrating system. Genome-integrating systems may deliver a genetically homogeneous cell population, independent from specific selection pressure for plasmid maintenance. However, the molecular cloning procedure for genome-integrating systems is very lengthy, time-consuming, and labour-intensive. We, applying cyanamide-inducible $^{I-}$*Sce*I expression, designed an *in-situ* promoter substitution system, which can significantly simplify the molecular cloning process (Fig. 2).

We constructed two plasmids, pILGFPEasy6R and pILGFPEasy8R. Both plasmids can be integrated into the yeast genome at the *ura3* locus, containing a *KlURA3* selectable marker, *yEGFP* gene fused with the *PGK*1 terminator, an $^{I-}$*Sce*I site, a G418-resistant selectable marker *KanMX4*, a *MAL32* promoter-controlled toxin gene *MazF* inserted with the *RPL28* intron. In plasmid pILGFPEasy8R, a E2Crimson reporter [50,51] fused with the *PDC1* terminator was inserted at the downstream of MazF and in the opposite direction of yEGFP gene. For *in situ* promoter substitution, the plasmid pILGFPEasy6R or pILGFPEasy8R can be transformed into a background strain harbouring a cyanamide-inducible $^{I-}$*Sce*I expression cassette. Prior to yeast transformation, cyanamide is added to induce $^{I-}$*Sce*I expression, and the transformed promoter fragment can repair $^{I-}$*Sce*I-mediated DSBs through homologous recombination. The cell mixtures can be selected on YP-maltose agar. In theory, toxin MazF gene is induced by maltose, and only the cells with MazF replaced can form colonies. Furthermore, the inducer chemicals can be supplemented to activate the heterologous promoter for yEGFP fluorescence-assisted selection.

We further constructed another plasmid (pILGFPEasy9R; Supplementary Fig. 2; Addgene# 218291) by inserting the P_{DDI2}-$^{I-}$*SceI*-T_{synth3} expression cassette into the downstream of the *PDC1* terminator in plasmid pILGFPEasy8R. This plasmid can be used in the background strain that does not harbour a pre-transformed $^{I-}$*Sce*I expression cassette.

3.3. Characterisation of regulatory promoters in S. cerevisiae

To test efficiency of *in situ* promoter substitution, we characterised a set of regulatory promoters, which were regulated by either endogenous regulatory mechanisms or synthetic regulatory mechanisms. We constructed a yeast strain (strain oQR36o). In strain oQR36o, four heterologous expression cassettes were introduced at the *HO* locus: (1) an artificial transcription factor (TF) Zif268-hER-VP16^{A} (the fusion of Zif268 zinc finger DNA binding domain, estrogen sensing domain, and VP16 transactivating domain) was expressed under the control of the YPK2 promoter, a weak promoter; (2) an artificial TF LmrA-SV40^{NSL}-

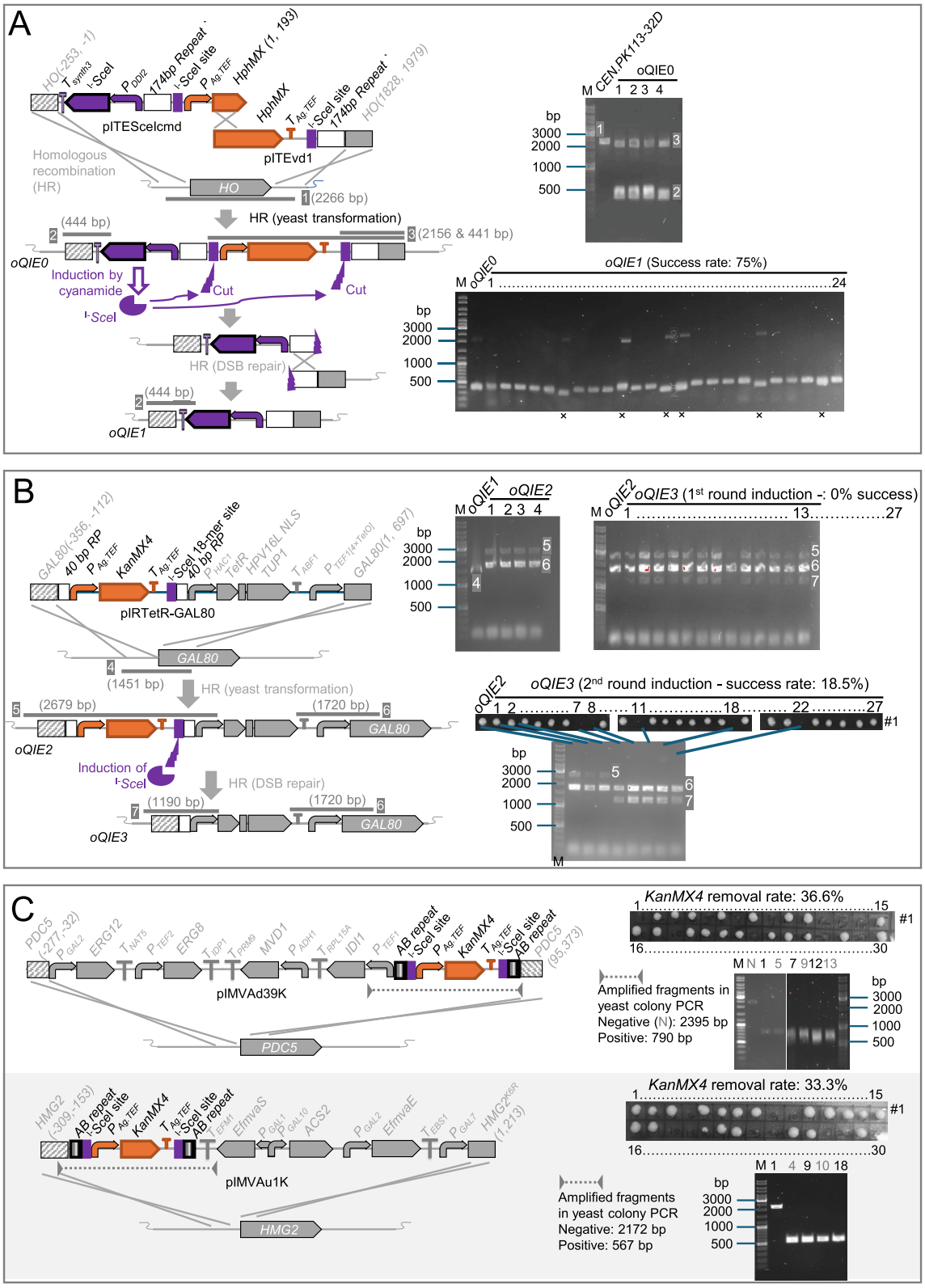

Fig. 1. Cyanamide-inducible expression of $^{I-}$*Sce*I facilitating selectable marker removal for iterative strain engineering. (A) Integration of cyanamide-inducible $^{I-}$*Sce*I cassette at *ho* locus and selectable marker removal. (B) Integration of tetracycline-inducible *GAL80* module for tetracycline-mediated repression on galactose-inducible promoters and selectable marker removal. (C) Integration of the mevalonate pathway modules and selectable marker removal. Negative, the *KanMX4* marker not being removed; Positive, the *KanMX4* marker being removed. P_{XXX}, the promoter of gene *XXX*; T_{XXX}, the terminator of gene *XXX*. T_{synth3}, a synthetic minimal terminator. NLS, nucleus-localising sequence. *TetR-HPV16L*^{NLS}*-TUP1*, encoding a tetracycline-depressible repressor. TetO, TetR-binding sequence. Metabolic pathway genes for terpene precursor synthesis: *ERG12*, *ERG8*, *MVD1*, *IDI1*, *EfmvaS*, *ACS2*, and *EfmvaE*. *GAL*, the genes involving in galactose metabolism and regulation. M, Thermo Scientific GeneRuler DNA Ladder Mix (Thermo Scientific#SM0331). #1, yeast clones replicated on the YPD agar supplemented with 300 μg ml^{-1} G418. The yeast colony PCR results are shown in DNA agarose gel images.

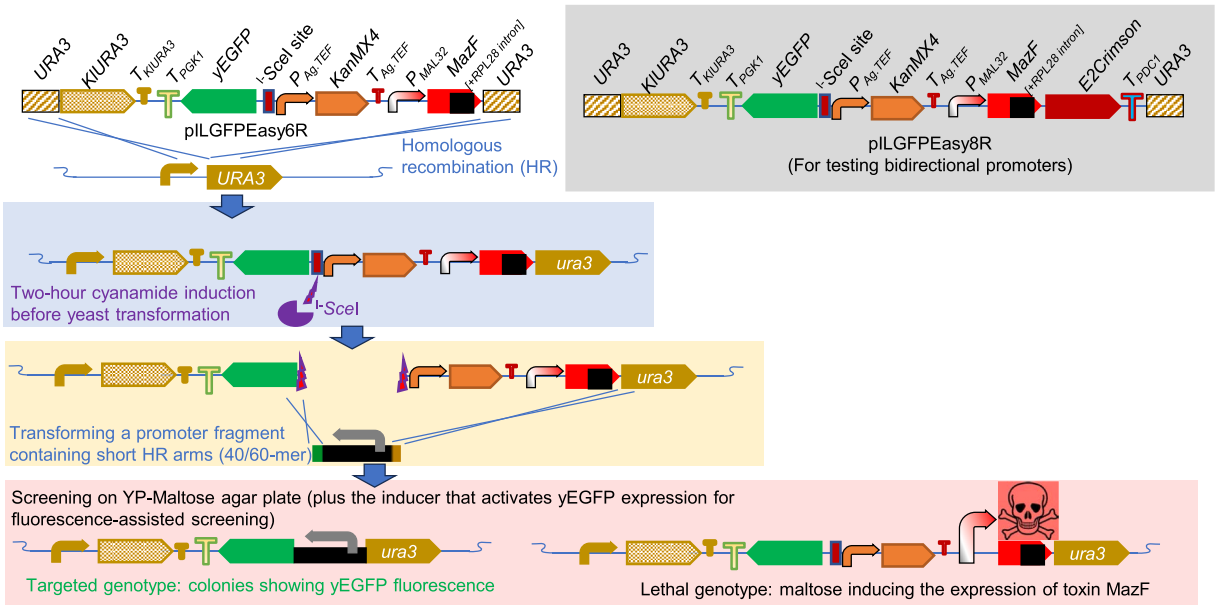

Fig. 2. Diagrams of *in situ* gene replacement design for plasmid-free cloning of heterologous promoters into yeast genome. MazF, encoding an *E. coli* interferase toxin.

Mig1^{C} (the fusion of a bacterial TetR-like TF LmrA, SV40 nucleus-localising sequence, and Mig1p C-terminal *trans*-repression domain) was expressed under the control of the *ADR1* promoter; (3) an artificial TF TetR-HPV16L^{NLS}-Tup1 (the fusion of tetracycline repressor TetR-HPV16L nucleus-localising sequence, and *trans*-repressing TF Tup1; TetR-Tup1) was expressed under the control of the *HAC1* promoter; and (4) $^{I-}$*SceI* was expressed under the control of cyanamide-inducible *DDI2* promoter.

Plasmid pILGFPEasy6R (linearised by *Pme*I) was transformed into strain oQR36o for characterisation of mono-directional promoters, and plasmid pILGFPEasy8R (linearised by *Pme*I) was transformed for characterisation of bidirectional promoters. Noted is that pILGFPEasy8R covers pILGFPEasy6R function. Each promoter fragment contains 40 or 60-bp homologous recombination arms for genomic integration. For yeast transformation, cells were spread to YP-maltose agar plates. At this step, it is recommended to spread 10 μl or less cells per plate, otherwise it can be difficult to isolate single colonies. Although brighter colonies were only a small proportion of those plated, they were found to be correctly transformed (Supplementary Fig. 1). Yeast colony PCR showed the successful promoter substitution. However, in *GAL10/1* promoter cases, it was difficult to isolate the single bright colonies from other non-fluorescent colonies on yeast transformation plates, which led to the amplification of two DNA fragments (Supplementary Fig. 1). Therefore, spreading less cells per plates is important for some cases. In the cases that we could not isolate the pure colonies from the transformation plates, the pure fluorescent colonies were isolated through streaking on fresh plates for further characterisation.

To test the use of pILGFPEasy9R, pILGFPEasy9R (linearised by *Pme*I) was transformed into strain CEN.PK113-5D. Using the resulting strain, we transformed *S. kudriavzevii GAL1* promoter [52]. Consistently, brighter colonies, although forming a smaller population, were verified using yeast colony PCR (Supplementary Fig. 2).

In total, we characterised five groups of inducible promoters. They are (1) tetracycline-inducible *TEF1[4×TetO]* promoters, (2) cyanamide-inducible *DDI2* promoter, (3) synthetic β-estradiol-inducible promoters, (4) maltose-inducible *MAL32/31* bidirectional promoters, and (5) five galactose-inducible *GAL10/1* promoters.

We previously synthesised a *TEF1[4×TetO]* promoter by inserting four *TetO* motifs between the core promoter and upstream activation sequence (UAS) of the *TEF1* promoter [38]. The *TEF1[4×TetO]* promoter is repressed in the strain expressing TetR-Tup1 and is inducible by tetracycline addition. The originally tested *TEF1[4×TetO]* promoter (Fig. 3A: UAS-579), containing a full-length UAS, demonstrates a moderate transcription strength. The tetracycline-inducible promoters with weaker strength might be useful to control the expression of certain genes, like the TF-encoding gene. We hypothesized that truncating UAS sequences might decrease promoter strength. We then tested two truncated *TEF1[4×TetO]* promoters, UAS-382 (the promoter starting at −382 position of the *TEF1* gene) and UAS-288 (the promoter starting at −288 position). Consistent with the hypothesis, truncated *TEF1[4×TetO]* promoters were weaker than the full-length one (Fig. 3A) by up to 62 %.

We characterised the *DDI2* promoter which was used to control $^{I-}$*SceI* expression in this study. In previous studies [40,41], the *DDI2* promoter was not characterised with an external reference. The *DDI2* promoter showed a strength lower than the *TEF1* promoter, causing ~6.4-fold decrease in yEGFP fluorescence (Fig. 3B).

Synthetic estrogen-inducible promoters were developed previously [53]. However, these promoters were leaky, i.e., for the *CYC1* core promoter fused with Zif268-binding elements (Fig. 3C) [38] and the *GAL1* promoter inserted with Zif268-binding elements [39]. To solve this problem, we hypothesized that an artificial *trans*-repressor could be used to repress its basal expression. Here, we tested a synthetic *CYC1*core promoter fused with four Z268 (Zif268-binding) elements and one LmrA-binding LmrO sequence (*CYC1core[4×Z268 + 1×LmrO]*). The *CYC1core[4×Z268 + 1×LmrO]* showed a very low basal expression and was inducible by estrogen β-estradiol. But the induction level was lower than that of the *CYC1core[4×Z268]* promoter, shown by ~4.7-fold decrease in yEGFP fluorescence (Fig. 3C).

For bidirectional promoters, we characterised maltose-inducible *MAL32/31* promoters and the galactose-inducible *GAL1/10* promoters from five *Saccharomyces* species: *S. cerevisiae* (*ScGAL10/1*), *S. arboricola* (*SaGAL1/10*), *S. kudriavzevii* (*SkGAL1/10*), *S. mikatae* (*SmGAL1/10*), and *S. paradoxus* (*SpdGAL1/10*). The *MAL32/31* and Sc*GAL1/10* promoters have been characterised for bidirectional transcriptional activation and strength previously [52]. For the *SaGAL1/10*, *SkGAL1/10*, *SmGAL1/10*, and *SpdGAL1/10* promoters, we previously measured the strength at the *GAL1* direction, and the *GAL10* promoters were not tested. We therefore used our system to re-characterise their activities. As the external reference, the strength of the *TEF1* promoter was characterised on glucose using E2Crimson, which was under the control of the *PGK1* terminator (Fig. 3D & E: dashed line). Consistent with the

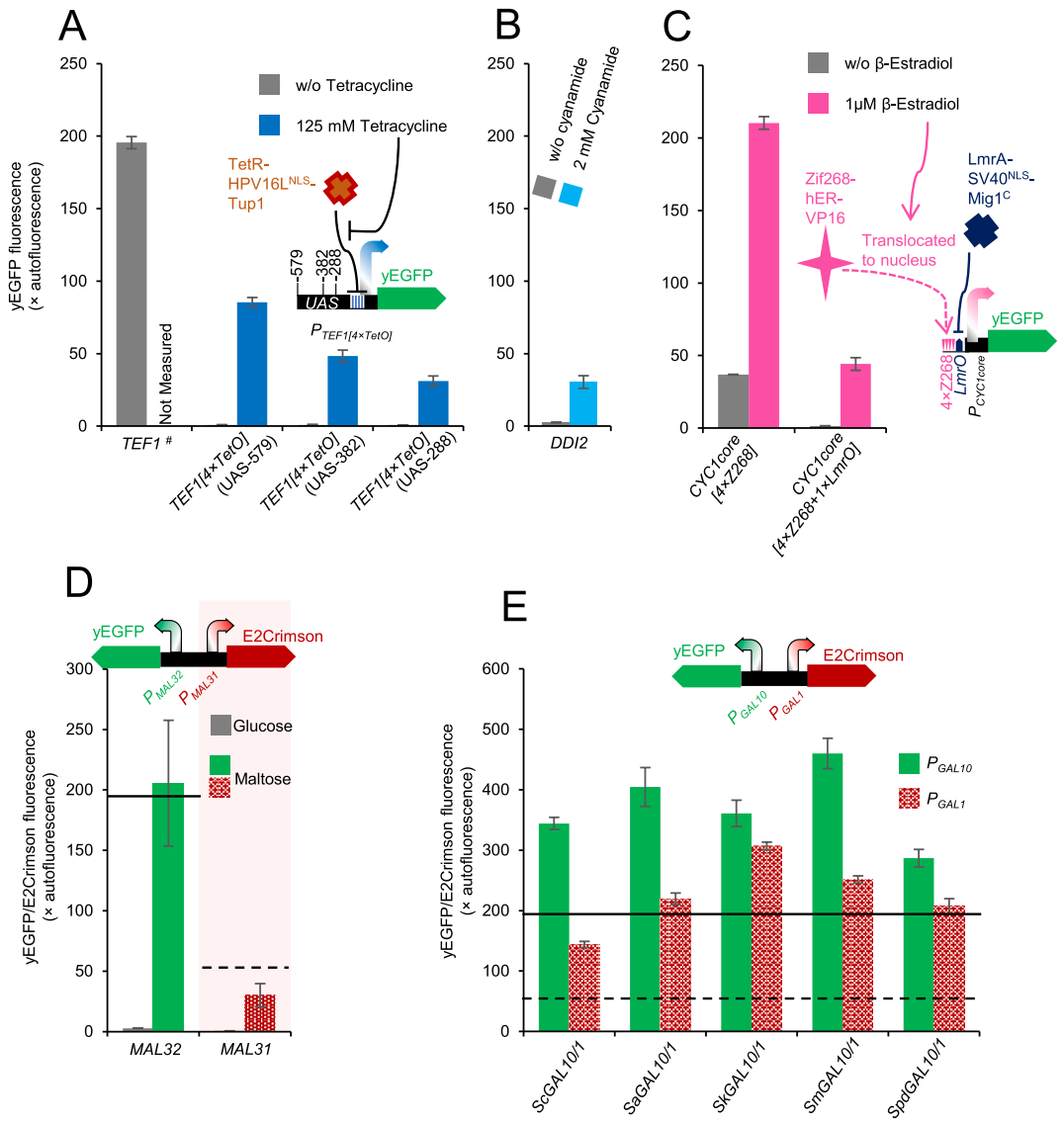

Fig. 3. Characterisation of regulatory promoters in *S. cerevisiae*. (A) Tetracycline-inducible *TEF1[4×TetO]* promoters: truncating the upstream activation sequence (UAS) resulted in decreased expression outputs. TetR-HPV16L^{NLS}-Tup1, an artificial *trans*-repressor binds to TetO sequences. Tetracycline binding to TetR disassociates the TetR-TetO interaction. (B) Cyanamide-inducible *DDI2* promoter. (3) Estrogen (β-estrodiol)-inducible promoters. Zif268-hER-VP16, an artificial *trans*-activator targeting to Z268 sequences. β-Estrodiol binding results in nuclear translocation of hER (human estrogen receptor). LmrA-SV40^{NSL}-Mig1^{C}, an artificial *trans*-repressor binding to the *LmrO* sequence. (D) Maltose-inducible promoters. (E) Galactose-inducible promoters. (D & E) The bold lines show the fluorescence level of yEGFP under the control of the *TEF1* promoter, and dashed lines were of E2Crimson. Cells were grown in YNB-glucose media (A and C), YP-glucose media (B & D), YP-maltose media (D), or YNB-galactose media (E). Mean values ± standard deviation were shown (n = 3 biological replicates).

previous study, both sides of the *MAL32/31* promoter were induced by maltose. The *MAL32* promoter was induced to the similar level of the *TEF1* promoter, and the *MAL31* promoter was at the halved level the *TEF1* promoter.

The *GAL1/GAL10* promoters were characterised on galactose in a *GAL80* wildtype background. Using E2Crimson as the reporter, the output from the *ScGAL1* promoter was ~2.6-fold of that from the *TEF1* promoter. This fold-change was in the same range with the ~2.5 fold in our previous measurement when yEGFP was used as the reporter [49]. For the *GAL1* promoters, the strength was sequenced as: *SkGAL1* > *SmGAL1* > *SaGAL1/SpdGAL1* > *ScGAL1*. All five *GAL10* promoters were stronger than the *TEF1* promoter. The strongest *GAL10* promoter was the *SmGAL10* promoter. The *SpdGAL10* promoter was the weakest *GAL10* promoter with the activity higher than the *TEF1* promoter by 50 %. These suggest that these five bidirectional *GAL1/10* promoters are good options for gene overexpression in metabolic engineering.

4. Discussion

In this study, we tested two yeast engineering methods for selectable marker removal and the plasmid-free promoter characterisation. Although not showing superior engineering efficiency, both methods can practically facilitate routine yeast engineering. Applying cyanamide-mediated gene induction allows its compatibility with pathway engineering using the galactose-inducible promoters. The *in-situ* promoter substitution system minimises the operational steps for characterisation of yeast promoters through genome integration. Both systems can be further improved and may be replicable in other non-conventional yeast platforms.

The cyanamide-inducible *DDI2* promoter was used in the aim to deliver orthogonal control on different genetic engineering tools. However, in comparison to the marker removal system applying the *GAL1* promoter to control $^{I-}$*Sce*I expression [47], the system applying the *DDI2* promoter showed a lower marker removal efficiency. This

might be due to that the strength of the *DDI2* promoter is lower than the strength of the *GAL1* promoter by ~17 folds (Fig. 3). In further development, alternative small-molecule-inducible genetic circuits can be considered, i.e., the ligand/factor pairs of 2,4-diacetylphloroglucinol/PhlF [54], camphor/CamR [54], 1,2-bis(4-hydroxyphenyl) ethane-1,2-dione/hER mutant [55], steroids/mammalian type I nuclear receptors [56]. These inducible circuits may not only be useful to control alternative meganucleases or genome-engineering tools, but also alternative killing toxins (like MazF; Fig. 3) for negative selections.

Nevertheless, it might be necessary to optimise the regulatory promoters for a tight ON/OFF response and minimise the promoter lengths [57] to facilitate PCR-based cloning and assembly. We have previously identified that the fusion of 4 × Z268-containing DNA sequence to the *CYC1* core promoter contributed to the basal expression [38], possibly due to endogenous *trans*-activators activating the transcription. Here, we solved the leakiness problem in a β-estradiol-inducible genetic circuit by introduction of a synthetic *trans*-repression mechanism in the regulatory promoter (Fig. 3C). The high affinity of a synthetic *trans*-activator to its target binding sites might also result in the leaky *trans*-activation [58]. To solve this problem, using a low-affinity DNA-binding domain eliminated the leakiness at the expense of the induction strength. To increase the induction output strength in this case, a molecular clamp was used to stabilise the interaction of multiple synthetic *trans*--activators and target DNA motifs at the target promoter. In the tetracycline-inducible circuit case, we showed that the length of upstream activation sequence (UAS) determined the induction strength (Fig. 3A). Consistently, tandem assembly of multi-UASs has been used to engineer super strong yeast promoters [59]. These emphasise that there are alternative solutions in synthetic biology to achieve similar biological effects and genetic circuit design principles shall be discovered for precise genetic design.

In summary, we prototyped the cyanamide-induced meganuclease tools to facilitate *in situ* genome engineering in *S. cerevisiae*. These tools may facilitate strain engineering at multiple genome loci and characterisation of yeast promoters. Availability of multiple fine small-molecule-inducible genetic circuits are important to develop more complexed networks for precise control on more genome engineering tools in a single cell [60,61]. In this scenario, it will be also important to develop computer software so that individual genome-engineering tools may work synergistically in a biofoundry setting to power the engineering biology capacity for synthetic biology and metabolic engineering applications [62].

CRediT authorship contribution statement

Liam McDonnell: Investigation, Methodology, Validation, Writing – review & editing, Formal analysis. **Samuel Evans:** Formal analysis, Investigation, Methodology, Validation, Writing – review & editing. **Zeyu Lu:** Methodology, Resources, Supervision. **Mitch Suchoronczak:** Investigation. **Jonah Leighton:** Investigation. **Eugene Ordeniza:** Investigation. **Blake Ritchie:** Investigation. **Nik Valado:** Investigation. **Niamh Walsh:** Investigation. **James Antoney:** Investigation, Resources. **Chengqiang Wang:** Conceptualization, Formal analysis. **Carlos Horacio Luna-Flores:** Conceptualization, Formal analysis, Resources. **Colin Scott:** Conceptualization, Resources. **Robert Speight:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision. **Claudia E. Vickers:** Conceptualization, Funding acquisition, Resources, Supervision. **Bingyin Peng:** Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft.

Declaration of competing interest

Authors declare no conflicts of interest.

Acknowledgements

This research was supported by the Australian Government through the Australian Research Council Centres of Excellence funding scheme (project CE200100029). The views expressed herein are those of the authors and are not necessarily those of the Australian Government or Australian Research Council.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2024.06.009.

References

[1] Lu Z, Shen Q, Liu L, Talbo G, Speight R, Trau M, et al. Profiling proteomic responses to hexokinase-II depletion in terpene-producing Saccharomyces cerevisiae. Eng Microb 2023;3(3):100079. https://doi.org/10.1016/j.engmic.2023.100079.
[2] Sun X, Yuan Y, Chen Q, Nie S, Guo J, Ou Z, et al. Metabolic pathway assembly using docking domains from type I cis-AT polyketide synthases. Nat Commun 2022;13(1):5541. https://doi.org/10.1038/s41467-022-33272-2.
[3] Yuan B, Wang W-B, Wang Y-T, Zhao X-Q. Regulatory mechanisms underlying yeast chemical stress response and development of robust strains for bioproduction. Curr Opin Biotech 2024;86:103072. https://doi.org/10.1016/j.copbio.2024.103072.
[4] Bureau JA, Oliva ME, Dong Y, Ignea C. Engineering yeast for the production of plant terpenoids using synthetic biology approaches. Nat Prod Rep 2023;40(12):1822–48. https://doi.org/10.1039/d3np00005b.
[5] Peng B, Weintraub SJ, Lu Z, Evans S, Shen Q, McDonnell L, et al. Integration of yeast episomal/Integrative plasmid causes genotypic and phenotypic Diversity and improved sesquiterpene production in Metabolically engineered Saccharomyces cerevisiae. ACS Synth Biol 2024;13(1):141–56. https://doi.org/10.1021/acssynbio.3c00363.
[6] Sun L, Zheng P, Sun J, Wendisch VF, Wang Y. Genome-scale CRISPRi screening: a powerful tool in engineering microbiology. Eng Microb 2023;3(3):100089. https://doi.org/10.1016/j.engmic.2023.100089.
[7] Chen Y, Lian J, Hou J. Metabolic engineering: tools and applications. Eng Microb 2023;3(4):100126. https://doi.org/10.1016/j.engmic.2023.100126.
[8] Chao R, Mishra S, Si T, Zhao H. Engineering biological systems using automated biofoundries. Metab Eng 2017;42:98–108. https://doi.org/10.1016/j.ymben.2017.06.003.
[9] Collins JH, Keating KW, Jones TR, Balaji S, Marsan CB, Çomo M, et al. Engineered yeast genomes accurately assembled from pure and mixed samples. Nat Commun 2021;12(1):1485. https://doi.org/10.1038/s41467-021-21656-9.
[10] Saa PA, Zaparte S, Drovandi CC, Nielsen LK. LooplessFluxSampler: an efficient toolbox for sampling the loopless flux solution space of metabolic models. BMC Bioinf 2024;25(1):3. https://doi.org/10.1186/s12859-023-05616-2.
[11] Enghiad B, Xue P, Singh N, Boob AG, Shi C, Petrov VA, et al. PlasmidMaker is a versatile, automated, and high throughput end-to-end platform for plasmid construction. Nat Commun 2022;13(1):2697. https://doi.org/10.1038/s41467-022-30355-y.
[12] Rapp JT, Bremer BJ, Romero PA. Self-driving laboratories to autonomously navigate the protein fitness landscape. Nat Chem Eng 2024;1(1):97–107. https://doi.org/10.1038/s44286-023-00002-4.
[13] Hicks JB, Hinnen A, Fink GR. Properties of yeast transformation. Cold Spring Harbor Symp Quant Biol 1979;43(Pt 2):1305–13. https://doi.org/10.1101/sqb.1979.043.01.149.
[14] Peng B, Esquirol L, Lu Z, Shen Q, Cheah LC, Howard CB, et al. An in vivo gene amplification system for high level expression in Saccharomyces cerevisiae. Nat Commun 2022;13(1):2895. https://doi.org/10.1038/s41467-022-30529-8.
[15] Evans S, Lu Z, McDonnell L, Anderson W, Peralta F, Watkins T, et al. The integration of tandem gene repeats via a bacterial type-II toxin-antitoxin-mediated gene amplification (ToxAmp) system and stability visualisation in Saccharomyces cerevisiae</em&gt. bioRxiv 2024;2024. https://doi.org/10.1101/2024.01.30.578080. 01.30.578080.
[16] Zhu Y, Liu J, Sun L, Liu M, Qi Q, Hou J. Development of genetic markers in Yarrowia lipolytica. Appl Microbiol Biotechnol 2024;108(1):14. https://doi.org/10.1007/s00253-023-12835-3.
[17] Hartzog PE, Nicholson BP, McCusker JH. Cytosine deaminase MX cassettes as positive/negative selectable markers in Saccharomyces cerevisiae. Yeast 2005;22(10):789–98. https://doi.org/10.1002/yea.1245.
[18] Liu Q, Liu H, Yang Y, Zhang X, Bai Y, Qiao M, et al. Scarless gene deletion using mazF as a new counter-selection marker and an improved deletion cassette assembly method in Saccharomyces cerevisiae. J Gen Appl Microbiol 2014;60:89–93. https://doi.org/10.2323/jgam.60.89.
[19] Kuijpers NG, Chroumpi S, Vos T, Solis-Escalante D, Bosman L, Pronk JT, et al. One-step assembly and targeted integration of multigene constructs assisted by the I-SceI meganuclease in Saccharomyces cerevisiae. FEMS Yeast Res 2013;13(8):769–81. https://doi.org/10.1111/1567-1364.12087.
[20] Holkenbrink C, Dam MI, Kildegaard KR, Beder J, Dahlin J, Doménech Belda D, et al. EasyCloneYALI: CRISPR/Cas9-Based synthetic toolbox for engineering of the yeast Yarrowia lipolytica. Biotechnol J 2018;13(9):1700543. https://doi.org/10.1002/biot.201700543.

[21] Siddiqui MS, Choksi A, Smolke CD. A system for multilocus chromosomal integration and transformation-free selection marker rescue. FEMS Yeast Res 2014;14(8):1171–85. https://doi.org/10.1111/1567-1364.12210.
[22] Bartley BA, Beal J, Karr JR, Strychalski EA. Organizing genome engineering for the gigabase scale. Nat Commun 2020;11(1):689. https://doi.org/10.1038/s41467-020-14314-z.
[23] Xu X, Meier F, Blount BA, Pretorius IS, Ellis T, Paulsen IT, et al. Trimming the genomic fat: minimising and re-functionalising genomes using synthetic biology. Nat Commun 2023;14(1):1984. https://doi.org/10.1038/s41467-023-37748-7.
[24] Wu X, Cai P, Yao L, Zhou YJ. Genetic tools for metabolic engineering of Pichia pastoris. Eng Microb 2023;3(4):100094. https://doi.org/10.1016/j.engmic.2023.100094.
[25] Ploessl D, Zhao Y, Cao M, Ghosh S, Lopez C, Sayadi M, et al. A repackaged CRISPR platform increases homology-directed repair for yeast engineering. Nat Chem Biol 2022;18(1):38–46. https://doi.org/10.1038/s41589-021-00893-5.
[26] Jelicic M, Schmitt LT, Paszkowski-Rogacz M, Walder A, Schubert N, Hoersten J, et al. Discovery and characterization of novel Cre-type tyrosine site-specific recombinases for advanced genome engineering. Nucleic Acids Res 2023;51(10):5285–97. https://doi.org/10.1093/nar/gkad366.
[27] Teng Y, Jiang T, Yan Y. The expanded CRISPR toolbox for constructing microbial cell factories. Trends Biotechnol 2023;42(1):104–18. https://doi.org/10.1016/j.tibtech.2023.06.012.
[28] Solis-Escalante D, Kuijpers NGA, van der Linden FH, Pronk JT, Daran J-M, Daran-Lapujade P. Efficient simultaneous excision of multiple selectable marker cassettes using I-SceI-induced double-strand DNA breaks in Saccharomyces cerevisiae. FEMS Yeast Res 2014;14(5):741–54. https://doi.org/10.1111/1567-1364.12162.
[29] Merrick CA, Zhao J, Rosser SJ. Serine Integrases: Advancing synthetic biology. ACS Synth Biol 2018;7(2):299–310. https://doi.org/10.1021/acssynbio.7b00308.
[30] Swidah R, Auxillos J, Liu W, Jones S, Chan TF, Dai J, et al. SCRaMbLE-in: a Fast and efficient method to Diversify and improve the Yields of heterologous pathways in synthetic yeast. Methods Mol Biol 2020;2205:305–27. https://doi.org/10.1007/978-1-0716-0908-8_17.
[31] Shaw WM, Studená L, Roy K, Hapeta P, McCarty NS, Graham AE, et al. Inducible expression of large gRNA arrays for multiplexed CRISPRai applications. Nat Commun 2022;13(1):4984. https://doi.org/10.1038/s41467-022-32603-7.
[32] Heidersbach AJ, Dorighi KM, Gomez JA, Jacobi AM, Haley B. A versatile, high-efficiency platform for CRISPR-based gene activation. Nat Commun 2023;14(1):902. https://doi.org/10.1038/s41467-023-36452-w.
[33] Jia B, Wu Y, Li B-Z, Mitchell LA, Liu H, Pan S, et al. Precise control of SCRaMbLE in synthetic haploid and diploid yeast. Nat Commun 2018;9(1):1933. https://doi.org/10.1038/s41467-018-03084-4.
[34] Güldener U, Heck S, Fielder T, Beinhauer J, Hegemann JH. A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic Acids Res 1996;24(13):2519–24. https://doi.org/10.1093/nar/24.13.2519.
[35] Hayat IF, Plan M, Ebert BE, Dumsday G, Vickers CE, Peng B. Auxin-mediated induction of GAL promoters by conditional degradation of Mig1p improves sesquiterpene production in Saccharomyces cerevisiae with engineered acetyl-CoA synthesis. Microbiol Biotech 2021;14(6):2627–42. https://doi.org/10.1111/1751-7915.13880.
[36] Zhou P, Fang X, Xu N, Yao Z, Xie W, Ye L. Development of a highly efficient Copper-inducible GAL regulation system (CuIGR) in Saccharomyces cerevisiae. ACS Synth Biol 2021;10(12):3435–44. https://doi.org/10.1021/acssynbio.1c00378.
[37] Wu Y, Deng J, Zheng Z, Chen N, Luo X, Tang H. Engineering an efficient expression using heterologous GAL promoters and transcriptional activators in Saccharomyces cerevisiae. ACS Synth Biol 2023;12(6):1859–67. https://doi.org/10.1021/acssynbio.3c00243.
[38] Peng B, Bandari NC, Lu Z, Howard CB, Scott C, Trau M, et al. Engineering eukaryote-like regulatory circuits to expand artificial control mechanisms for metabolic engineering in Saccharomyces cerevisiae. Commun Biol 2022;5(1):135. https://doi.org/10.1038/s42003-022-03070-z.
[39] Shaw WM, Khalil AS, Ellis T. A Multiplex MoClo Toolkit for extensive and flexible engineering of Saccharomyces cerevisiae. ACS Synth Biol 2023;12(11):3393–405. https://doi.org/10.1021/acssynbio.3c00423.
[40] Lin A, Zeng C, Wang Q, Zhang W, Li M, Hanna M, et al. Utilization of a strongly inducible DDI2 promoter to control gene expression in Saccharomyces cerevisiae. Front Microbiol 2018;9:2736. https://doi.org/10.3389/fmicb.2018.02736.
[41] Wang Y, Zhang K, Li H, Xu X, Xue H, Wang P, et al. Fine-tuning the expression of target genes using a DDI2 promoter gene switch in budding yeast. Sci Rep-UK 2019;9(1):12538. https://doi.org/10.1038/s41598-019-49000-8.
[42] Meurer M, Chevyreva V, Cerulus B, Knop M. The regulatable MAL32 promoter in Saccharomyces cerevisiae: characteristics and tools to facilitate its use. Yeast 2017;34(1):39–49. https://doi.org/10.1002/yea.3214.
[43] Gietz RD, Schiestl RH. High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. Nat Protoc 2007;2(1):31–4. https://doi.org/10.1038/nprot.2007.13.
[44] Cormack BP, Bertram G, Egerton M, Gow NAR, Falkow S, Brown AJP. Yeast-enhanced green fluorescent protein (yEGFP): a reporter of gene expression in Candida albicans. Microb (Reading) 1997;143(Pt 2):303–11. https://doi.org/10.1099/00221287-143-2-303.
[45] Strack RL, Hein B, Bhattacharyya D, Hell SW, Keenan RJ, Glick BS. A rapidly maturing far-red derivative of DsRed-Express2 for whole-cell labeling. Biochemistry-US 2009;48(35):8279–81. https://doi.org/10.1021/bi900870u.
[46] Entian K-D, Kötter P. 23 yeast mutant and plasmid collections. Methods in microbiology. Elsevier; 1998. p. 431–49.
[47] Peng B, Plan MR, Carpenter A, Nielsen LK, Vickers CE. Coupling gene regulatory patterns to bioprocess conditions to optimize synthetic metabolic modules for improved sesquiterpene production in yeast. Biotechnol Biofuels 2017;10(1):43. https://doi.org/10.1186/s13068-017-0728-x.
[48] Stephanopoulos G. Synthetic biology and metabolic engineering. ACS Synth Biol 2012;1(11):514–25. https://doi.org/10.1021/sb300094q.
[49] Peng B, Williams TC, Henry M, Nielsen LK, Vickers CE. Controlling heterologous gene expression inyeast cell factories on different carbon substrates and across the diauxic shift: acomparison of yeast promoter activities. Microb Cell Fact 2015;14(1):91. https://doi.org/10.1186/s12934-015-0278-5.
[50] Strack RL, Hein B, Bhattacharyya D, Hell SW, Keenan RJ, Glick BS. A rapidly maturing far-red derivative of DsRed-Express2 for whole-cell labeling. Biochemistry-US 2009;48(35):8279–81. https://doi.org/10.1021/bi900870u.
[51] Lu Z, Shen Q, Bandari NC, Evans S, McDonnell L, Liu L, et al. LowTempGAL: a highly responsive low temperatureinducible *GAL* system in Saccharomyces cerevisiae. Nucleic Acids Res 2024. https://doi.org/10.1093/nar/gkae460.
[52] Peng B, Wood RJ, Nielsen LK, Vickers CE. An expanded heterologous GAL promoter collection for Diauxie-inducible expression in Saccharomyces cerevisiae. ACS Synth Biol 2018;7(2):748–51. https://doi.org/10.1021/acssynbio.7b00355.
[53] Rantasalo A, Kuivanen J, Penttilä M, Jäntti J, Mojzita D. Synthetic Toolkit for complex genetic circuit engineering in Saccharomyces cerevisiae. ACS Synth Biol 2018;7(6):1573–87. https://doi.org/10.1021/acssynbio.8b00076.
[54] Tominaga M, Nozaki K, Umeno D, Ishii J, Kondo A. Robust and flexible platform for directed evolution of yeast genetic switches. Nat Commun 2021;12(1):1846. https://doi.org/10.1038/s41467-021-22134-y.
[55] Chockalingam K, Chen Z, Katzenellenbogen JA, Zhao H. Directed evolution of specific receptor-ligand pairs for use in the creation of gene switches. Proc Natl Acad Sci U S A. 2005;102(16):5691–6. https://doi.org/10.1073/pnas.0409206102.
[56] Sanford A, Kiriakov S, Khalil AS. A Toolkit for precise, multigene control in Saccharomyces cerevisiae. ACS Synth Biol 2022;11(12):3912–20. https://doi.org/10.1021/acssynbio.2c00423.
[57] Redden H, Alper HS. The development and characterization of synthetic minimal yeast promoters. Nat Commun 2015;6(1):7810. https://doi.org/10.1038/ncomms8810.
[58] Bragdon MDJ, Patel N, Chuang J, Levien E, Bashor CJ, Khalil AS. Cooperative assembly confers regulatory specificity and long-term genetic circuit stability. Cell 2023;186(18):3810. https://doi.org/10.1016/j.cell.2023.07.012. 25.e18.
[59] Deng J, Wu Y, Zheng Z, Chen N, Luo X, Tang H, et al. A synthetic promoter system for well-controlled protein expression with different carbon sources in Saccharomyces cerevisiae. Microb Cell Fact 2021;20(1):202. https://doi.org/10.1186/s12934-021-01691-3.
[60] Cummins B, Vrana J, Moseley RC, Eramian H, Deckard A, Fontanarrosa P, et al. Robustness and reproducibility of simple and complex synthetic logic circuit designs using a DBTL loop. Synth Biol 2023;8(1). https://doi.org/10.1093/synbio/ysad005. Article ysad005.
[61] Mante J, Myers CJ. Advancing reuse of genetic parts: progress and remaining challenges. Nat Commun 2023;14(1):2953. https://doi.org/10.1038/s41467-023-38791-0.
[62] Densmore D, Hillson NJ, Klavins E, Myers C, Peccoud J, Stracquadanio G. Introduction to the special Issue on BioFoundries and Cloud laboratories. J Emerg Technol Comput Syst 2023;19(3). https://doi.org/10.1145/3609485. Article 26.