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RESEARCH ARTICLE

Development of a dual temperature control system for isoprene biosynthesis in *Saccharomyces cerevisiae*

Jiaxi Lin[1], Zhen Yao[3], Xiaomei Lyu[4], Lidan Ye (✉)[1,2], Hongwei Yu (✉)[1]

1 Institute of Bioengineering, College of Chemical and Biological Engineering, Zhejiang University, Hangzhou 310027, China
2 Hangzhou Global Scientific and Technological Innovation Center, Zhejiang University, Hangzhou 311200, China
3 Key Laboratory of Systems Microbial Biotechnology, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China
4 School of Food Science and Technology, Jiangnan University, Wuxi 214122, China

Abstract Conflict between cell growth and product accumulation is frequently encountered in the biosynthesis of secondary metabolites. To address the growth-production conflict in yeast strains harboring the isoprene synthetic pathway in the mitochondria, the dynamic control of isoprene biosynthesis was explored. A dual temperature regulation system was developed through engineering and expression regulation of the transcriptional activator Gal4p. A cold-sensitive mutant, Gal4ep19, was created by directed evolution of Gal4p based on an internally developed growth-based high-throughput screening method and expressed under the heat-shock promoter P_{SSA4} to control the expression of P_{GAL}-driven pathway genes in the mitochondria. Compared to the control strain with constitutively expressed wild-type Gal4p, the dual temperature regulation strategy led to 34.5% and 72% improvements in cell growth and isoprene production, respectively. This study reports the creation of the first cold-sensitive variants of Gal4p by directed evolution and provides a dual temperature control system for yeast engineering that may also be conducive to the biosynthesis of other high-value natural products.

Keywords transcriptional activator, directed evolution, dynamic control, heat-shock, isoprene

1 Introduction

Isoprene, as an important feedstock for the synthetic chemical industry and a building block of terpenes, has become a target product for many synthetic biologists [1]. For the efficient biosynthesis of isoprene in *Saccharomyces cerevisiae*, we developed a cytoplasmic/mitochondrial dual regulation strategy to maximize acetyl-CoA utilization [2], which in combination with the engineered isoprene synthase resulted in the production of 11.9 $g \cdot L^{-1}$ isoprene [3]. In particular, localization of the P_{GAL}-driven synthetic pathway in acetyl-CoA-rich mitochondria was demonstrated to be efficient in enhancing isoprene production. Considering that the expression level of *GAL4* is generally low and that the low amount of transcriptional activator may limit the biosynthetic efficiency of the multigene pathway under the control of *GAL* promoters, enhancing the supply of Gal4p may be a viable approach to further improving isoprene synthesis. In fact, Gal4p overexpression significantly promoted isoprene synthesis when the pathway was localized in the cytoplasm [4]. However, when *GAL4* was overexpressed in the strain harboring the mitochondrial isoprene synthetic pathway, growth impairment was observed, implying excessive metabolic burden (Fig. S1, cf. Electronic Supplementary Material, ESM). The adverse effect of isoprene biosynthesis on yeast cells may be explained by the competition of precursors (acetyl-CoA) and energy (adenosine 5′-triphosphate (ATP), nicotinamide adenine dinucleotide phosphate) during simultaneous cell growth and product accumulation [5]. Therefore, the growth-production conflict is one of the major barriers to the further improvement of isoprene production in mitochondrial engineered yeast.

Numerous efforts have been made to develop metabolic regulation strategies for controllable expression of the synthetic pathway. Galactose-mediated induction of gene expression in yeast under control of the transcriptional activator Gal4p is the best characterized native regulation system in *S. cerevisiae* [6,7]. By knocking out *GAL80*, it can be modified to a glucose-responsive system [8]. However, this system is limited by imprecise regulation

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E-mails: yelidan@zju.edu.cn (Ye L), yuhongwei@zju.edu.cn (Yu H)

and leaky expression [8]. As a readily controllable fermentation parameter, temperature is a more ideal input signal for the design of stringent control systems. Temperature-sensitive (ts) mutants of Gal4p that show heat sensitivity and behave like the wild type (WT) strain at temperatures below the restrictive temperature have been created by the insertion of conditionally splicing inteins [9,10] and protein engineering [11–14] and could be used as potential switches for the expression control of pathway enzymes with optimal temperatures lower than the growth temperature. Insertion of a ts mutant of the vacuolar ATPase subunit, TS19, which is self-cleaved at 18 °C and loses its self-shearing activity at 29 °C, into Gal4 generated the ts transcription factor Gal4INT^{TS19}. Site-directed mutagenesis of putatively buried hydrophobic residues created ts mutants of Gal4 that were active at 21 °C and almost inactive at 30 °C or above [11]. In the above examples, the ts Gal4p variants were used to regulate the expression of single genes such as *GFP*, *LacZ*, *HIS3*, *ADE2*, or *URA3*. For efficient regulation of multi-gene pathways, we recently created the ts Gal4M9 mutant by directed evolution based on an internally developed lycopene-based high-throughput screening method and introduced it into *GAL80*-deleted yeast to create a temperature-responsive regulation system, leading to successful separation of growth and production stages in lycopene fermentation [13].

In contrast with carotenogenic enzymes, which favor a relatively low temperature, isoprene synthase, as the rate-limiting enzyme in isoprene biosynthesis, shows an optimum temperature of approximately 37 °C (Fig. S2, cf. ESM). Development of a temperature-responsive regulatory system for such pathways would require cold-sensitive (cs) Gal4p mutants instead. Unlike ts mutants, cs mutants show lower activity or even behave like loss-of-function mutants at temperatures lower than a cutoff temperature but have WT-like phenotypes at higher temperatures. The lack of sufficient understanding of the structure-function relationship of Gal4p discourages its rational design, while directed evolution requires a proper high-throughput screening method. The previously developed lycopene-based method was not suitable for screening cs mutants since carotenogenesis is limited at 37 °C (Fig. S3, cf. ESM). In *S. cerevisiae*, 5-fluoro-orotic acid (5-FOA) has long been used in the counterselection of *URA3*$^+$ cells [15–17] due to the cytotoxicity of its conversion product 5-fluorouridine. By placing *URA3* under the control of Gal4p and screening on 5-FOA-containing medium, cs mutants of Gal4p may be selected with growth rate as an indicator.

Temperature-responsive expression of transcription factors represents an alternative strategy for the construction of temperature-controlled systems. By expressing the ts mutants of Gal4p with dramatically decreased activity in a heat-responsive manner, the expression of P_{GAL}-driven genes exhibited a cs phenotype instead [18]. In that system, the coupling of partial loss-of-function Gal4p mutants with a heat-shock promoter delivered almost no regulation activity at temperatures lower than the cutoff temperature and showed activity at higher temperatures due to the difference in the expression levels. If a cs Gal4p mutant could be expressed under the control of a heat-shock promoter, its regulatory activity at the permissive temperature would be further increased due to the enhanced expression level, and the basal expression of P_{GAL}-driven pathway genes at the restrictive temperature would be minimized (Fig. 1).

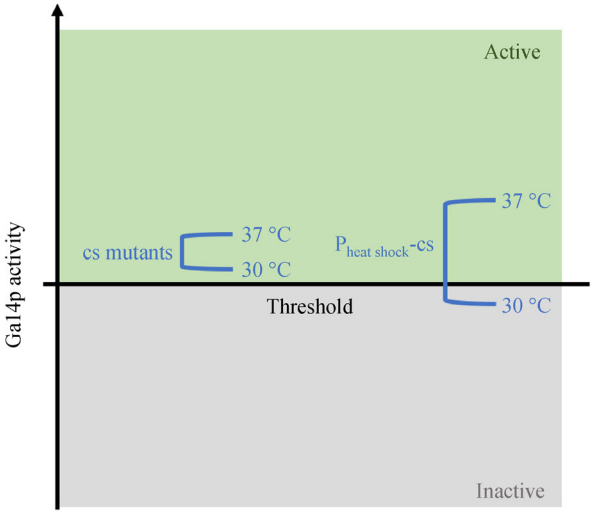

Fig. 1 Model for a dual temperature control system constructed by heat-responsive expression of a cs Gal4p mutant. The cs Gal4p mutant activity was lower at 30 °C than at 37 °C. For the cs Gal4p mutant under the control of a heat-shock promoter, the activity at 37 °C was amplified, while that at 30 °C was further reduced and even fell below the threshold (detection limit).

Herein, with the aim of solving the conflict between cell growth and isoprene synthesis from a mitochondrial pathway, we developed a growth-based high-throughput screening method based on *URA3*/5-FOA, created cs mutants of Gal4p by directed evolution, and then coupled the Gal4p mutant with a heat-shock promoter to establish a conditional and reversible control system with 37 °C as the permissive temperature and 30 °C as the restrictive temperature. This novel temperature-responsive regulation system was then applied to control the isoprene synthetic pathway in mitochondrial engineered yeast, leading to obviously improved cell growth and isoprene production. The temperature control system developed would provide a useful tool for the expression regulation of pathway enzymes with optimum temperatures higher than 30 °C in yeast.

2 Experimental

2.1 Construction of plasmids and strains

The *GAL4* knockout cassette was composed of a *LEU2* selection marker and the upstream and downstream homologous regions of the *GAL4* gene, and it was constructed by overlap extension polymerase chain reaction (PCR). The recombinant yeast strains used in this study were derived from *S. cerevisiae* BY4741 [19]. The details of strain construction are listed in Table S1 (cf. ESM). For directed evolution of Gal4p, p416XWP-*MET*-P_{ERG9}-*GAL4* plasmids containing the *MET15* selection marker were constructed based on p416XWP-P_{ERG9}-*GAL4* [13]. *Sac* II-*MET15*-*Nhe* I was generated by PCR amplification of MET15*Sac*II-R and MET15*Nhe*I-F using the BY4742 genome as the template, and the PCR products were cloned into the *Sac* II and *Nhe* I sites of p416XWP-P_{ERG9}-*GAL4*. For the generation of *GAL4* mutants, random mutagenesis of *GAL4* was conducted by PCR using p416XWP-P_{ERG9}-*GAL4* as the template, and the PCR products were cloned into the *Not* I and *Sac* I sites of p416XWP-*MET*-P_{ERG9}-*GAL4* using a one-step cloning kit (Vazyme, Nanjing, China). P_{GAL1}-P_{ERG9} and P_{SSA4}-P_{GAL1} bidirectional promoters were inserted into the respective sites of p416XWP01 via overlap extension PCR, generating p416XWP01-P_{ERG9} and p416XWP01-P_{SSA4}, respectively. *MLS-ISPSLN* [3] was then cloned into the *Sal*I and *Hind* III sites of the above constructs to generate p416XWP01-*MLSLN*-P_{ERG9} and p416XWP01-*MLSLN*-P_{ERG9}. Subsequent insertion of the *GAL4* fragment into the *Not* I and *Sac* I sites of these plasmids generated p416XWP01-*MLSLN-GAL4WT* and p416XWP01-*MLSLN*-P_{SSA4}-*GAL4WT* for isoprene fermentation.

For characterization of heat-shock promoters, P_{SSA4}, $P_{SSA4\text{-}1}$, and $P_{SSA4\text{-}2}$ were amplified from the BY4741 genome using primer pairs P1HSE-NOTI-R/QUETA-BamH1-F, P1HSE-NOTI-R/BAMH-1HSE-F and P1HSE-NOTI-R/BAMH-2HSE-F, respectively. The amplification products were then inserted into the corresponding sites of pESC-*URA* together with *Eco*R I-*EGFP*-*Sac* I, generating pESC-*URA*-*SSA4*-*EGFP*, pESC-*URA*-1HSE-*EGFP* and pESC-*URA*-2HSE-*EGFP*. Similarly, the control construct pESC-URA-P_{ERG9}-*EGFP* was built by inserting *Nhe* I-P_{ERG9}-*Not* I and *Eco*R I-*EGFP*-*Sac* I into pESC-*URA*. For further analysis of the temperature-responsive regulation capability of P_{SSA4}, P_{ERG9}-*GAL4WT* and P_{SSA4}-*GAL4WT* were inserted into pESC-*URA*, generating pESC-*URA*-*SSA4*-*EGFP* and pESC-*URA*-*ERG9*-*EGFP*, respectively.

Plasmids were transformed into *S. cerevisiae* via the LiAc/single-stranded carrier DNA/polyethylene glycol method [20]. *Escherichia coli* Top 10 cells were used for cloning. All primers (synthesized by Sangon Biotech, Shanghai, China) and recombinant plasmids used in this paper are listed in Tables S2 and S3 (cf. ESM).

2.2 Media and culture conditions in sealed vials

Yeast extract peptone dextrose medium (2% peptone, 1% yeast extract and 2% D-glucose) was used for the routine cultivation of yeast. Synthetic dextrose (SD, synthetic complete drop-out medium with 2% D-glucose) was used for the selection and cultivation of yeast carrying plasmids with the corresponding auxotrophic marker. SD-FOA (SD medium with 0.1% w/v of 5-FOA) was used for the high-throughput screening of yeast strains.

Due to the low boiling point of isoprene (34 °C), shake-flask experiments had to be conducted in sealed vials. Single yeast colonies were picked into 5 mL of SD-URA medium and cultivated at 30 °C and 220 $r \cdot min^{-1}$ for 24 h. The seed broth was then inoculated into 17 mL sealed vials containing 3 mL SD-URA medium to an initial OD_{600} (optical density) of 0.05 and incubated at 30 °C or 37 °C and 220 $r \cdot min^{-1}$ for 48 h. When incubated at 30 °C, the culture was heated to 37 °C (220 $r \cdot min^{-1}$) for 30 min after cultivation to vaporize isoprene for gas chromatography (GC) quantification. All yeast strains were transformed with low-copy-number plasmids derived from p416XWP01-*MLSLN*-P_{ERG9}.

2.3 GC analysis of isoprene

Isoprene was quantified on a GC system (Fuli, Wenling, China) equipped with an HP-FFAP column (30 m × 0.25 mm, 0.25 μm, Agilent) and a flame ionization detector. N_2 served as the carrier gas. The temperatures of the oven, detector and injector were 80 °C, 180 °C and 180 °C, respectively. Two hundred microliters of sample was taken from the headspace of sealed vials or the off-gas of the fermenter and injected into the GC system. The isoprene standard was purchased from Aladdin (Shanghai, China).

2.4 High-performance liquid chromatography (HPLC) analysis of glucose

Glucose was measured at 45 °C on an HPLC system equipped with a refractive-index detector (LC-20AT, Shimadzu) and a Bio-Rad HPX-87H column using 5 $mmol \cdot L^{-1}$ H_2SO_4 as the mobile phase at a flow rate of 0.6 $mL \cdot min^{-1}$.

2.5 Fluorescence intensity measurement

Y113EGFP-0, Y113EGFP-1, Y113EGFP-2, and Y113EGFP-S were inoculated to an initial OD_{600} of 0.05 and incubated at 30 °C or 37 °C and 220 $r \cdot min^{-1}$ for 96 h. The cells from 2 mL of each sample were collected by centrifugation and washed twice with cold phosphate buffer (100 $mmol \cdot L^{-1}$, pH = 7.0). The cells were then resuspended in the abovementioned phosphate buffer to OD_{600} = 1 for fluorescence measurements using a microplate reader (INFIN-200Pro, Tecan I-Control

2.0.10.0). The excitation wavelength and emission wavelength were 488 and 530 nm, respectively.

2.6 Real-time quantitative PCR (qPCR) analysis

Total RNA was isolated from yeast cells by the RNAiso Plus Kit (Takara, Dalian, China) according to the protocol. Genomic DNA contamination in the RNA samples was eliminated by DNase I (Takara, Dalian, China). Reverse transcription was conducted using the PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara, Dalian, China) with oligo dT primers. Real-time qPCR was performed with PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, USA) on a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, USA). The *ACT1* gene was used as the internal control for normalization across different samples. The transcriptional levels of the corresponding genes were analyzed using the $2^{-\Delta\Delta CT}$ method [21].

2.7 Batch fermentation in a 5 L bioreactor

The medium used in batch fermentations consisted of 25 $g \cdot L^{-1}$ glucose, 15 $g \cdot L^{-1}$ $(NH_4)_2SO_4$, 8 $g \cdot L^{-1}$ KH_2PO_4, 3 $g \cdot L^{-1}$ $MgSO_4$, 0.72 $g \cdot L^{-1}$ $ZnSO_4 \cdot 7H_2O$, 12 $mL \cdot L^{-1}$ vitamin solution [22] and 10 $mL \cdot L^{-1}$ trace element solution [22]. Single colonies were placed into 5 mL of SD-URA medium, incubated at 30 °C for 24 h, and then transferred to three flasks containing 100 mL of fermentation medium to an initial OD_{600} of 0.05. After 24 h, 300 mL of the seed culture was inoculated into a 5 L bioreactor containing 2.5 L of fermentation medium for batch fermentation. Fermentation in the bioreactor was conducted at 30 °C and 400 $r \cdot min^{-1}$, with an air flow rate of 1–3 vvm and an overpressure of 0.05 MPa, and the pH was maintained at 5.0 by the automatic addition of ammonium hydroxide. Off-gas and fermentation broth were sampled for analysis at intervals.

3 Results and discussion

3.1 Directed evolution for cs Gal4p

A previous study [3] found that the cell growth of isoprene-producing strains was accompanied by the accumulation and release of isoprene. However, isoprene biosynthesis via a mitochondria-compartmented pathway presents a severe burden for the cells [2], possibly due to competition for acetyl-CoA. This growth-production puzzle inspired us to develop a switch for turning on pathway expression after biomass accumulation. After deletion of the *GAL80* gene, Gal4p becomes the sole controller for the transcription of P_{GAL}-controlled genes, which was therefore selected for switch development. First, a growth-based high-throughput screening method was constructed for the directed evolution of Gal4p toward acquired temperature sensitivity (Fig. 2). The 5′-phosphate orotate nucleoside decarboxylase encoded by *URA3* plays a key role in converting 5-FOA into 5-fluorouridine, which is a pyrimidine analog that can replace uracil or thymine in RNA and DNA synthesis, interfere with the processing and function of RNA and DNA, and cause cell death (Fig. 2(a)) [15,23]. Therefore, by expressing the *URA3* gene under the control of the *GAL* promoter, a negative correlation between Gal4p activity and the biomass of 5-fluorouridine-accumulating strains could be established. Transformants that grew on SD-URA plates at 37 °C but not at 30 °C were selected as potential candidates. To avoid false positive results caused by uracil accumulation, gradient dilution was used for rescreening cs Gal4 mutants (Fig. 2(b)).

The 881-aa transcriptional activator Gal4p consists of a DNA binding domain (residues 1–106), two transcription activation domains (residues 148–196, residues 768–881) [24] and a domain of unknown function (DUF, covering residues 197–767) [13]. We chose the first-half sequence (1-1321 bp) of *GAL4* encoding the DNA binding domain, the first transcriptional activation region and part of the unpredicted region as the template to generate an error-prone PCR library for directed evolution. A total of 14 putative mutants were selected from the library using the growth-based high-throughput screening method, among which 4 were confirmed in rescreening after gradient dilution as positive cs mutants, namely, ep5, ep16, ep19 and ep21 (Fig. 3).

After knocking out endogenous *GAL4*, we introduced plasmids expressing the Gal4p mutants and the previously created isoprene synthase mutant *ISPSLN* [3] into the yeast strain M08H-CS harboring a complete mevalonic acid pathway in the mitochondria. All four mutants not only improved isoprene production but also increased the biomass (Fig. 4), implying the relief of metabolic burden. Among these mutants, Gal4ep19 showed the highest improvement in isoprene production (98%) compared to WT Gal4p.

Sequencing of these mutants revealed 4–6 mutation sites in each mutant. In ep5, ep16 and ep21, mutation sites were detected in all three regions, whereas ep19 was mutated only in the DUF (Table 1). To acquire a better understanding of the mechanism behind the cs behavior of Gal4ep19, single-site directed mutagenesis was performed to evaluate the influence of each mutation on its performance. Among the four mutation sites, T262A and R378G contributed to the enhancement of biomass, but in both cases, isoprene production was decreased (Fig. S4, cf. ESM). Mutant N308D increased isoprene production by 71.5% compared to the WT, which explained approximately 58% of the production improvement by ep19. These results implied a synergistic effect of these mutation sites. Although no specific function has been assigned to the DUF domain, it was found to play a possibly important role in the temperature sensitivity of Gal4p. A previous

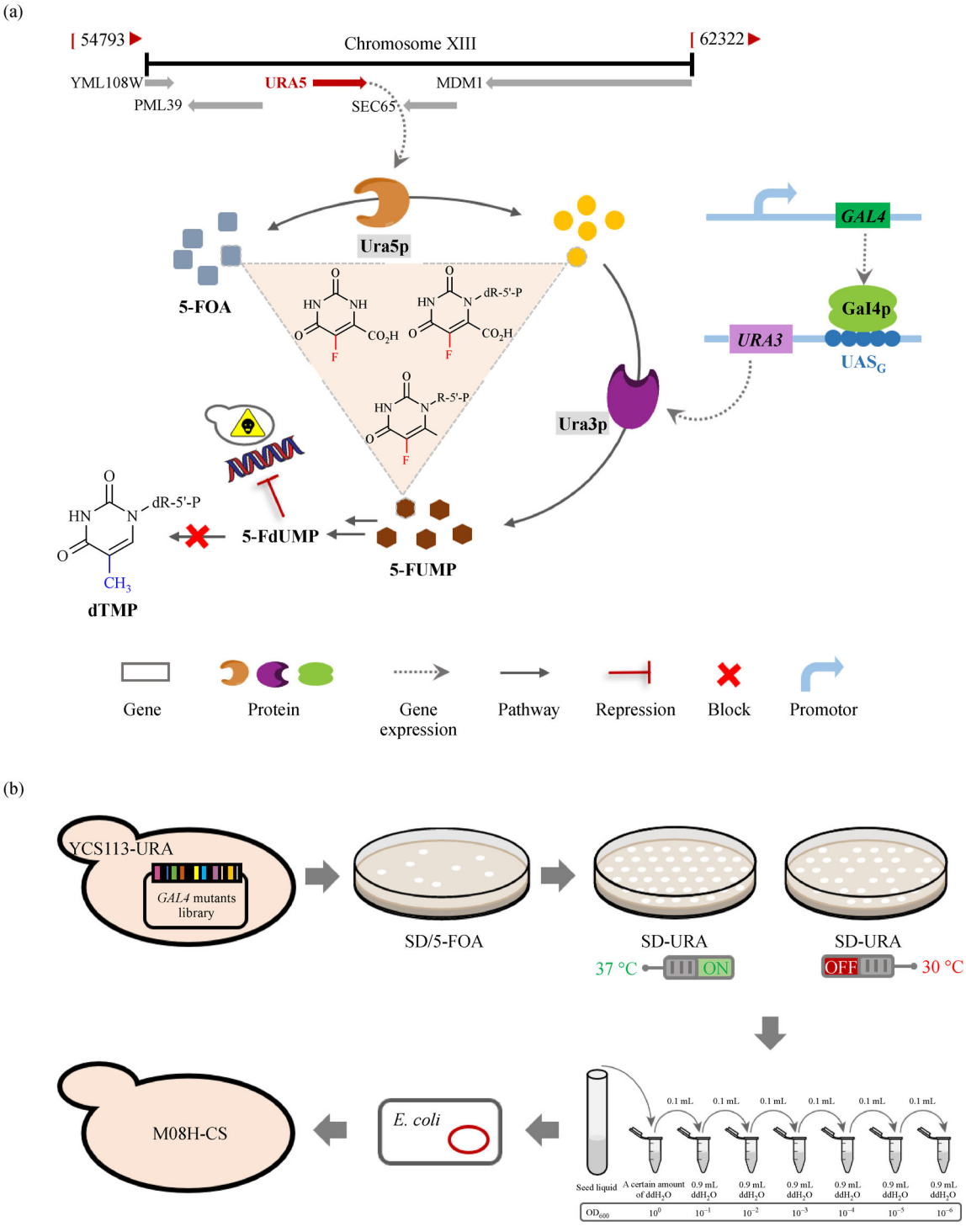

Fig. 2 Strategy and flowchart of Gal4p-directed evolution. (a) Principle of the 5-FOA/*URA3* cytotoxicity-based screening method. 5-FOA could be catalyzed into cytotoxic 5-fluorouridine by Ura5p and Ura3p, resulting in cell death (Ura5p: orotate phosphoribosyltransferase; Ura3p: orotidine-5′-phosphate decarboxylase; 5-FUMP: 5-fluorouridine monophosphate; 5-FdUMP: 5-fluorodeoxyuridine monophosphate; dTMP: deoxythymidine monophosphate). (b) Flowchart of Gal4p directed evolution. A mutant library was constructed by error-prone PCR. The transformants were first incubated on SD/5-FOA plates at 30 °C and then photoprinted onto two SD-URA plates: one was cultured at 30 °C, and the other was cultured at 37 °C. Colonies grown at 37 °C but not at 30 °C were selected and subjected to gradient dilution. Five microliters of diluted cells were spotted on SD plates lacking uracil. Positive variants were selected for further verification in the isoprene producer M08H-CS after propagation in *E. coli*.

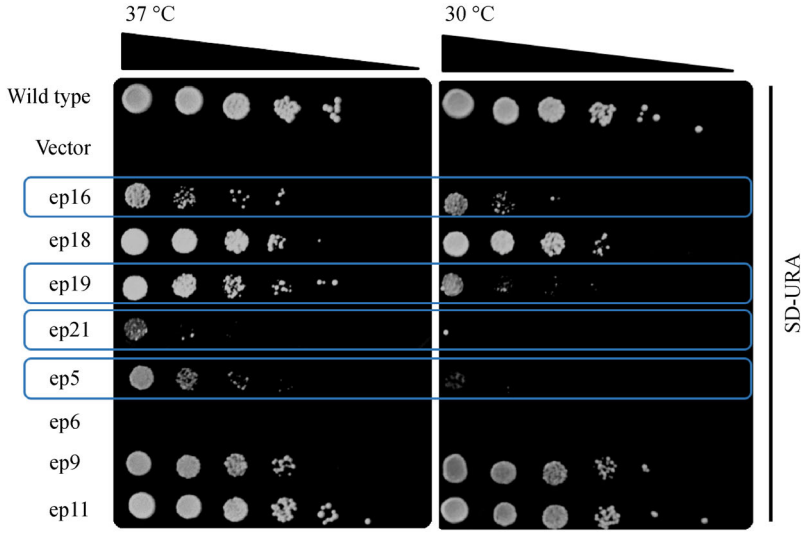

Fig. 3 Rescreening of potential cs Gal4p mutants. YCS113-URA cells transformed with WT Gal4p and the corresponding mutant were cultured on SD-URA. Triangles represent serial 10-fold dilutions, and the starting concentration was 1 OD_{600}. Colonies that grew well at 37 °C but not at 30 °C or grew badly at 30 °C were considered positive mutants (indicated by blue squares).

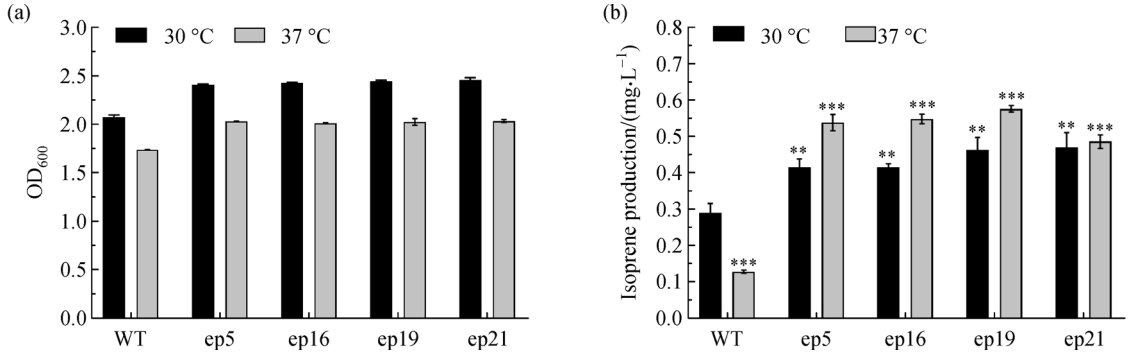

Fig. 4 Isoprene production and biomass of strains harboring WT and mutant Gal4p. (a) Comparison of the biomass at 30 °C and 37 °C among the cs Gal4p mutants and WT Gal4p; (b) comparison of isoprene production at 30 °C and 37 °C among the Gal4p cs mutants and WT Gal4p (Cells were cultured in sealed vials. The data presented are the means of three biological replicates. Error bars represent the standard deviations. Significant levels of the *t*-test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Table 1 Mutation sites of the Gal4 mutants

Mutants [a)]	DNA binding domain		DNA activation domain			DUF			
ep5	H53H	K82E	A152A	W174R	L187P				P423L
ep16	D12G	L81L		E176G				F211L	S219S
ep19		–		–		T262A	M265T	N308D	R378G
ep21	K25E			L184S		K212K	Q270R	S350R	W383R

a) Majuscule represents amino acid changes in the Gal4 mutants.

study on the DUF of Gal4p suggested its function of assisting the C(6) zinc cluster in recognition of the target sequence [25]. Deletion of the DUF or alteration of its sequence was found to reduce the expression of the Gal1-lacZ fusion protein [26,27]. In addition, point mutations of the DUF (T406A, I407V, and V413A) led to improved Gal4p activity [28]. Moreover, ts Gal4p mutants such as M9 (L69P, A356T), M61 (V385D), and M414 (S208P) also had mutations in DUF [13]. Consistent with these studies, our results further supported the importance of the DUF in Gal4p activity and temperature sensitivity.

3.2 Heat-shock expression of Gal4p

As observed in previous protein engineering studies, including those on the creation of ts Gal4p mutants [11–13], trade-offs of temperature responsiveness and regulatory activity may also occur during the generation of cs Gal4p mutants. Due to the lack of understanding of the structure-activity relationship of Gal4p, it is difficult to carry out further modification of Gal4p, and thus, we explored the expression regulation of Gal4p to compensate for the activity reduction.

SSA4 is the only one of five heat-inducible HSP70 genes in *S. cerevisiae* that is expressed at extremely low levels under normal conditions and is rapidly and dramatically induced following heat-shock treatment. Hence, the *SSA4* promoter could serve as a complement to compensate for the activity reduction of the cs Gal4p mutant and increase the expression change upon a temperature shift (Fig. 5). The heat-inducible regulation of the *SSA4* promoter is mediated by conserved eukaryotic heat-shock elements (HSEs) located in the *SSA4* promoter (Fig. 5(a)), which function as heat shock factor (HSF) binding sites [29]. To investigate the effect of HSE number on the heat-shock response of the *SSA4* promoter, we designed *SSA4* promoter variants containing one, two and three HSE modules. Using the green fluorescent protein *EGFP* as the reporter, we found that the constitutive promoter P_{ERG9} was not affected by temperature, as expected, with equal fluorescence intensity at 30 °C and 37 °C. In the three HSE-harboring promoters, only P_{SSA4} containing three HSE modules exhibited a heat-shock effect. The fluorescence intensity for P_{SSA4}-controlled *EGFP* at 37 °C was 1.3 times higher than that at 30 °C, and the fluorescence intensity at 30 °C was 10% lower than that of constitutive P_{ERG9}. $P_{SSA4\text{-}1}$ and $P_{SSA4\text{-}2}$ containing one or two HSE modules did not show a heat-shock effect, and the fluorescence intensity was lower than that of P_{ERG9} (Fig. 5(b)). The heat-shock promoter P_{SSA4} was then used to regulate the expression of *GAL4WT*, and its activity at 30 °C and 37 °C was reported by P_{GAL}-driven *LacZ*, using P_{ERG9}-driven WT *GAL4* as a control. As shown in Fig. 5 (c), under the control of the *SSA4* promoter, the expression of WT Gal4p at 30 °C was very low, delivering hardly any blue product (5-bromo-4-chloroindigo), whereas its expression at 37 °C obviously increased to a level slightly higher than that of its P_{ERG9}-driven counterpart, as indicated by the obvious accumulation of 5-bromo-4-chloroindigo. These results confirmed the feasibility of using P_{SSA4} to construct a temperature control system by regulating the expression of Gal4p.

To further examine the effect of temperature control on isoprene production, we introduced the P_{SSA4}-*GAL4WT* and P_{ERG9}-*GAL4ep19* constructs into the mitochondrial engineered strain M08H-CS and determined isoprene production in shake-flask cultures constantly grown at 30 °C or 37 °C or with a temperature shift from 30 °C to 37 °C after 24 h. Compared to the control (P_{ERG9}-*GAL4WT*), the isoprene yield of the Gal4ep19 strain regulated by P_{ERG9} was increased by 99% at 30 °C/37 °C, whereas the isoprene production of the strain controlled by P_{SSA4}-driven WT Gal4p was slightly decreased (Fig. 6). To check whether there was synergy between the temperature-responsive variations in Gal4p activity and expression, a dual temperature control system was constructed by heat-responsive expression of the cs Gal4p mutant. The P_{SSA4}-regulated Gal4ep19 strain increased isoprene production by 132% (Fig. 6), which was superior to the single temperature control systems. The superiority of the dual temperature control system over the Gal4ep19-mediated single temperature control system and further over the P_{SSA4}-mediated single temperature control system in isoprene biosynthesis suggested the complex effect of transcriptional activator regulation on the cells. Only when its activity and amount were controlled to appropriate levels could biosynthesis be maximally improved. Similarly, it was previously observed that Gal4p overexpression driven by the relatively weaker P_{GAL4} led to greater improvement in isoprene production in the cytoplasmic engineered strain than did P_{GAL1}-driven Gal4p overexpression, although both strains produced more isoprene than the control strain without Gal4p overexpression [4].

3.3 Batch fermentation for isoprene production

Mitochondria are a superior subcellular compartment for isoprene production due to their rich stock of acetyl-CoA [30]. The generation of acetyl-CoA through the aerobic oxidation of carbohydrates requires abundant oxygen. However, due to its low boiling point, isoprene may be lost when the cells are aerobically cultured in shake flasks. To prevent product loss by evaporation, sealed serum bottles were used instead of normal conical flasks for shake-flask experiments, which limited the oxygen supply. To avoid the adverse effect of oxygen deficiency on the mitochondrial synthetic pathway, aerobic fermentation was performed for the temperature-controlled strains (Fig. 6(a)) (M08H-CS-MLN-SSA4-WT, M08H-CS-MLN-SSA4-ep19, M08H-CS-MLN-ep19) together with the control strain (M08H-CS-MLN-WT) in bioreactors.

First, the control strain M08H-CS-MLN-WT was cultured under different temperature conditions, namely, at a constant temperature of 30 °C or 37 °C or with a temperature shift from 30 °C to 37 °C. The highest production of isoprene was detected under constant culture at 30 °C, reaching 128 $mg \cdot L^{-1}$, with a maximum OD_{600} of 2.645 (Fig. S5, cf. ESM). The higher isoprene production at 30 °C than at 37 °C excluded the possibility that the production improvement under the control of temperature-responsive systems resulted from the higher isoprene synthase activity at 37 °C rather than the dynamic control of pathway expression.

All strains with temperature control were cultured with a

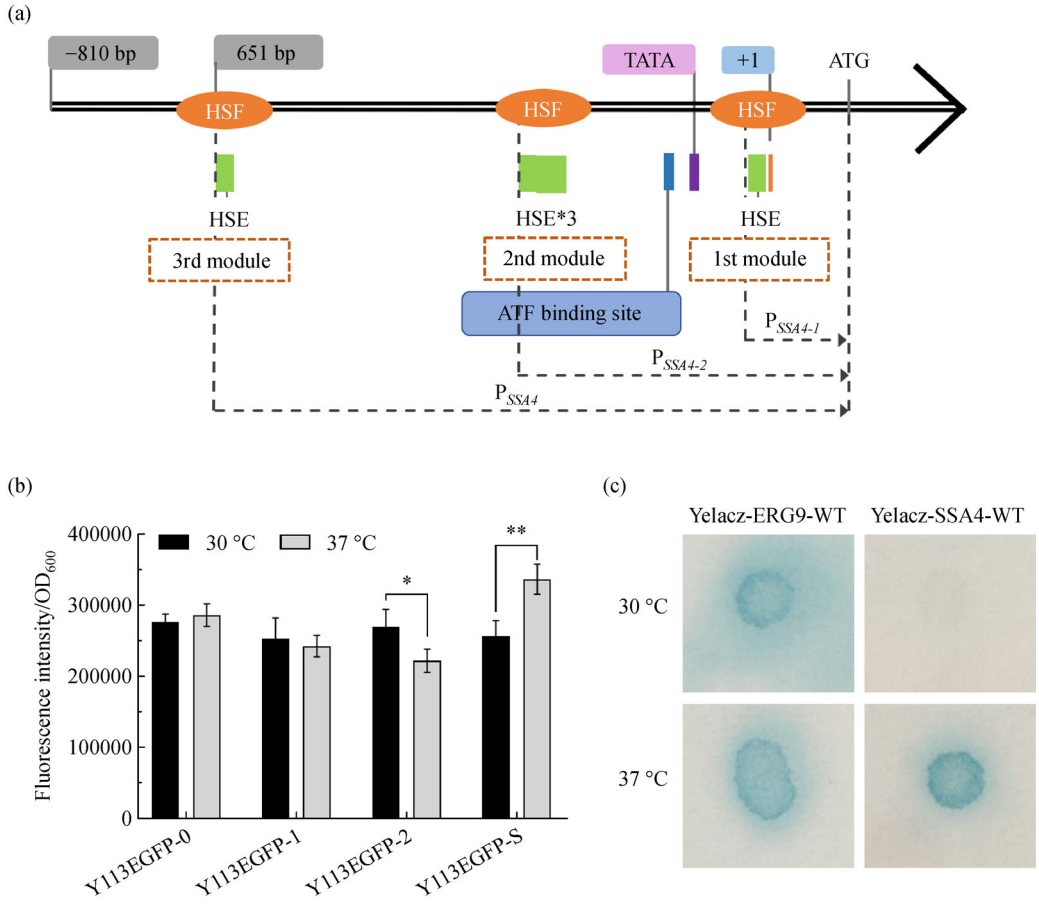

Fig. 5 Structure and characterization of the *SSA4* promoter: (a) Structure of the *SSA4* promoter. HSEs are essential elements for the heat response in the *SSA4* promoter. Under extreme conditions, HSFs are activated and bind tightly with HSE to initiate gene transcription. P_{SSA4-1}, P_{SSA4-2} and P_{SSA4} contain 1, 2 or 3 HSE modules, respectively (ATF: activating transcription factor). (b) Fluorescence characterization of heat-shock promoters of different lengths. Y113EGFP-0 served as a control, where the *EGFP* gene was regulated by the constitutive promoter P_{ERG9}, whereas *EGFP* in Y113EGFP-1, Y113EGFP-2, and Y113EGFP-S was regulated by P_{SSA4-1}, P_{SSA4-2}, and P_{SSA4}, respectively (The error bars represent standard deviations calculated from triplicate experiments). (c) Qualitative analysis of WT and mutant Gal4p under P_{SSA4} in yeast. P_{ERG9}-*GAL4WT* and P_{SSA4}-*GAL4WT* cassettes were carried on the pESC-*URA* plasmid and transformed into the Yelacz strain. Phenotypes were analyzed by monitoring UAS_G-*lacZ* reporter gene expression driven by WT Gal4p. The hydrolysis of X-Gal by *β*-gal yielded a blue-colored product. The blue color was used as a measure of the expression level of *β*-gal, which in turn represented the activity of Gal4p (The data presented are the means of three biological replicates. Error bars represent the standard deviations. Significant levels of the *t*-test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

temperature shift from 30 °C to 37 °C at late-log phase. Both strains with a single temperature control accumulated higher titers of isoprene than the nontemperature-regulated M08H-CS-MLN-WT strain. Strain M08H-CS-MLN-SSA4-WT produced 135 $mg \cdot L^{-1}$ isoprene, and its growth tendency was similar to that of the control strain (Fig. 7). M08H-CS-MLN-ep19 had a maximum OD_{600} of 3.09, implying alleviation of the metabolic burden compared to the control strain (Fig. 7(a)), and the glucose consumption rate was improved (Fig. S6, cf. ESM). Isoprene production eventually reached 186 $mg \cdot L^{-1}$ (Fig. 7(b)). This result implied that the temperature response of Gal4p activity led to better growth and higher production than its temperature-responsive expression. Cells of strain M08H-CS-MLN-SSA4-ep19 with dual temperature regulation grew faster than those of the other strains. In the first 20 h, M08H-CS-MLN-SSA4-ep19 grew to the highest OD_{600}, 3.6 (Fig. 7(a)). Before the temperature change at 15 h, the glucose utilization rate was 0.54 $g \cdot L^{-1} \cdot h^{-1}$, and within 12 h after the temperature change, the glucose utilization rate increased to 0.87 $g \cdot L^{-1} \cdot h^{-1}$ (Fig. S6). During this period, isoprene production increased exponentially, and the productivity of isoprene reached a maximum of 11.86 $mg \cdot L^{-1} \cdot h^{-1}$ (Fig. 7(c)) at 5 h after the temperature change.

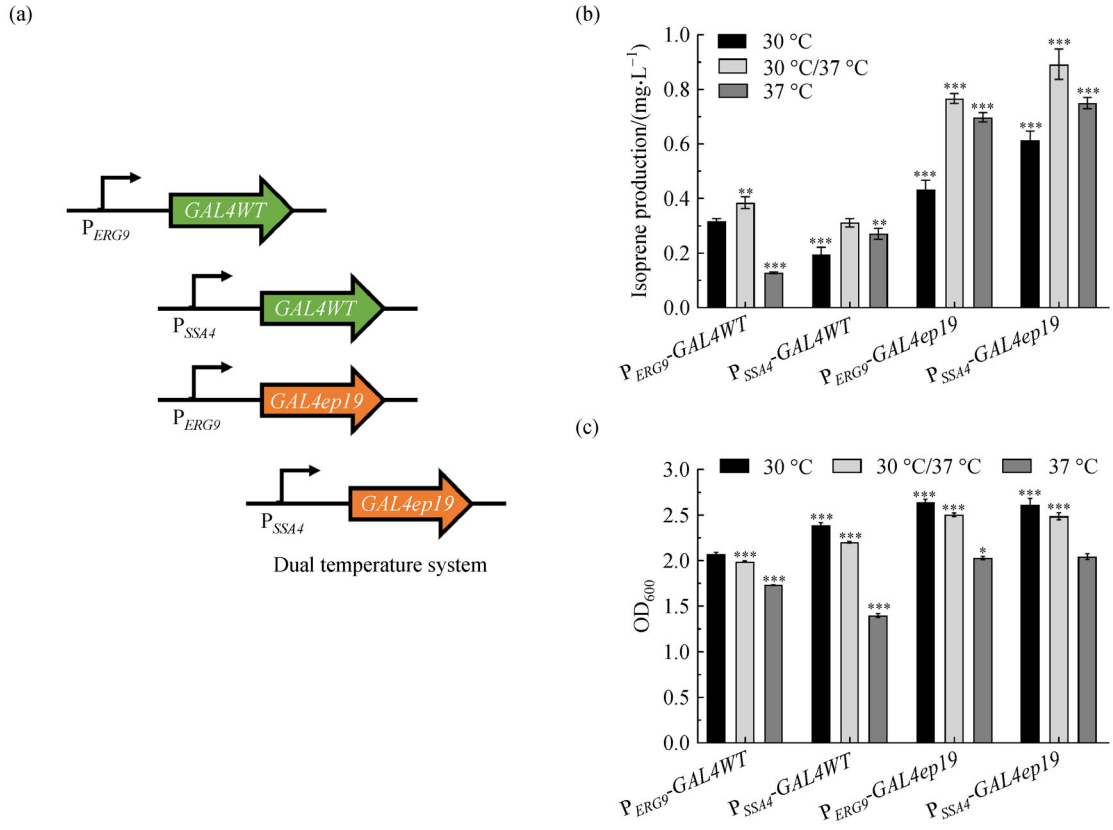

Fig. 6 Temperature control systems and their effects on isoprene production and yeast growth: (a) Schematic diagram of the temperature control systems. Control (constitutive expression of WT Gal4p): P_{ERG9}-*GAL4WT*; single temperature control system constructed by heat-shock promoter-mediated expression regulation of WT Gal4p or by Gal4p directed evolution: P_{SSA4}-*GAL4WT* and P_{ERG9}-*GAL4ep19*; dual temperature control system constructed by both directed evolution and expression regulation of Gal4p: P_{SSA4}-*GAL4ep19*. (b) Effect of the temperature control systems on isoprene production. (c) Effect of the temperature control systems on yeast growth. (Cells were cultured in sealed vials. The data presented are the means of three biological replicates. Error bars represent the standard deviations. Significant levels of *t*-test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

The final isoprene yield was significantly higher than that of M08H-CS-MLN-WT (220 mg·L^{-1} *vs.* 128 mg·L^{-1}) (Fig. 7(b)).

Transcriptional analysis showed obvious upregulation of Gal4ep19 under the control of P_{SSA4} after heat shock at 37 °C, and the transcriptional level of Gal4ep19 was increased by 59.6 times (Fig. 7(d)). However, the transcriptional levels of the P_{GAL}-controlled pathway genes exemplified by *tHMG1* and the isoprene synthase gene *ISPSLN* were not increased as much, with 16- and 6-fold upregulation, respectively (Fig. 7(d)). This result demonstrated that the temperature regulation system indeed improved isoprene production by controlling the expression of P_{GAL}-controlled pathway genes, but excessive Gal4p seemed to limit the increased amplitude of the expression of pathway genes. The regulation of Gal4p expression is therefore more challenging than we expected. Our result is consistent with the previous finding that excessive Gal4p could lead to suppression rather than a further increase in gene transcription [31]. Therefore, the fine-tuning of Gal4p overexpression would be a future direction for improving the developed dual temperature control system.

4 Conclusions

Although the molecular mechanism of cs Gal4 remains unclear, a Gal4 variant Gal4ep19 with a cs phenotype was acquired by directed evolution based on the internally developed growth-based high-throughput screening method and applied for the temperature control of isoprene biosynthesis in *S. cerevisiae*, leading to 98% production improvement. When expressed under the heat-shock promoter P_{SSA4}, Gal4ep19 delivered more than 1.32 times higher isoprene production than the control strain

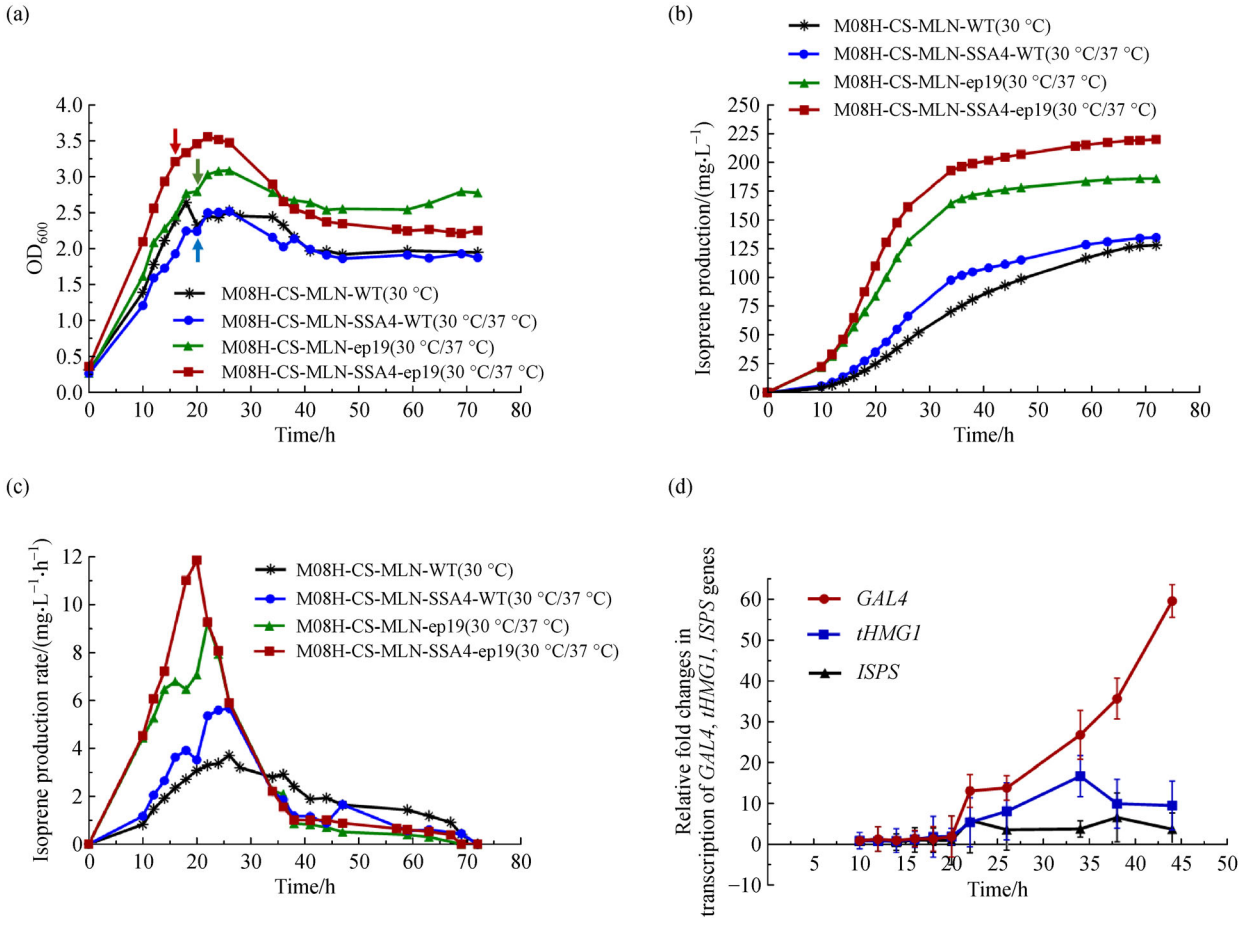

Fig. 7 Aerobic batch fermentation of isoprene by recombinant yeast strains and transcriptional levels of some genes in M08H-CS-MLN-SSA4-ep19 during fermentation: (a) Time course of the OD_{600}; (b) time course of isoprene production. The control strain M08H-CS-MLN-WT was cultured constantly at 30 °C, whereas the temperature-controlled strains M08H-CS-MLN-SSA4-WT, M08H-CS-MLN-ep19 and M08H-CS-MLN-SSA4-ep19 were cultured with a 30 °C /37 °C temperature shift at the late log-phase. The arrows indicate the time point of the temperature shift; (c) time course of isoprene productivity; (d) transcriptional levels of *GAL4*, *tHMG1*, and *ISPS* during the fermentation of M08H-CS-MLN-SSA4-ep19 (The error bars represent standard deviations calculated from triplicate experiments).

constitutively expressing WT Gal4. The aerobic fermentation results further confirmed the superior performance of the dual temperature control strain over the single temperature control strains and further over the nontemperature-regulated control, with both higher isoprene production and improved biomass. This work preliminarily explored the temperature regulation of isoprene synthesis in *S. cerevisiae* by combining engineering and expression regulation of the transcriptional activator Gal4p. This dual temperature regulation strategy provides helpful information for the design and construction of controllable biosynthetic pathways in yeast using temperature as an input signal.

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