Dynamic control of \textit{ERG9} expression for improved amorpha-4,11-diene production in \textit{Saccharomyces cerevisiae}

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Abstract

**Background:** To achieve high-level production of non-native isoprenoid products, it requires the metabolic flux to be diverted from the production of sterols to the heterologous metabolic reactions. However, there are limited tools for restricting metabolic flux towards ergosterol synthesis. In the present study, we explored dynamic control of \textit{ERG9} expression using different ergosterol-responsive promoters to improve the production of non-native isoprenoids.

**Results:** Several ergosterol-responsive promoters were identified using quantitative real-time PCR (qRT-PCR) analysis in an engineered strain with relatively high mevalonate pathway activity. We found mRNA levels for \textit{ERG11}, \textit{ERG2} and \textit{ERG3} expression were significantly lower in the engineered strain over the reference strain BY4742, indicating these genes are transcriptionally down-regulated when ergosterol is in excess. Further replacement of the native \textit{ERG9} promoter with these ergosterol-responsive promoters revealed that all engineered strains improved amorpha-4,11-diene by 2~5-fold over the reference strain with \textit{ERG9} under its native promoter. The best engineered strain with \textit{ERG9} under the control of \textit{P}_{\text{ERG1}} produced amorpha-4,11-diene to a titer around 350 mg/L after 96 h cultivation in shake-flasks.

**Conclusions:** We envision dynamic control at the branching step using feedback regulation at transcriptional level could serve as a generalized approach for redirecting the metabolic flux towards product-of-interest.

**Keywords:** Mevalonate pathway, Dynamic control, \textit{ERG9}, Ergosterol-responsive promoter, Isoprenoids, \textit{Saccharomyces cerevisiae}

Background

Microbial production of natural products in genetically tractable microbes has gained tremendous interest in the recent years. In order to produce these molecules at industrial levels, pathway genes involved in the synthesis of these molecules must be expressed at appropriately balanced levels, to avoid the accumulation of toxic intermediates or bottlenecks that result in growth inhibition or suboptimal yields [1-3]. Moreover, it often requires the metabolic flux towards side pathways to be minimized or completely eliminated [4-8]. For example, high-level production of non-native isoprenoid products requires the metabolic flux to be diverted from the production of ergosterol to the heterologous metabolic reactions. Down-regulation of \textit{ERG9} gene which encodes squalene synthase (the first committed step after farnesyl diphosphate in ergosterol biosynthesis), using the methionine-repressible \textit{MET3} promoter or copper-repressible \textit{CTR3} promoter, increased amorpha-4,11-diene production an additional 2-fold [9,10]. Other approaches such as harnessing weak promoter for controlling \textit{ERG9} expression and utilizing \textit{HXT1} promoter to couple \textit{ERG9} expression with glucose concentration also showed promising results [4,11].

As ergosterol fulfills several essential functions and each requires optimal sterol concentrations, synthesis of sterols in yeast is tightly regulated. In budding yeast, it requires thirteen-enzymatic steps to synthesize ergosterol from farnesyl diphosphate – a precursor from the mevalonate pathway (Figure 1). Previously, \textit{ERG9} expression in yeast was reported to be positively and negatively regulated by diverse factors such as the heme activator...
protein transcription factor HAP1/2/3/4 and the phospholipid transcription factor complex INO2/4 [12,13]. Sterol biosynthetic mutations at ERG3, ERG7 and ERG24 also increased ERG9 expression level. However, naturally occurring cognate regulator for ERG9 expression will rarely suffice to regulate an engineered pathway with higher metabolic flux. This leads us to search for other ergosterol-responsive promoters from ergosterol biosynthesis pathway (Table 1) for a better and tighter control of ERG9 expression, with further improved production of non-native isoprenoids. Previous investigations revealed that genes involved in ergosterol biosynthesis pathway such as ERG1, ERG11, ERG2 and ERG3 were transcriptionally up-regulated when yeast cells are treated with inhibitors to restrict metabolic flux towards ergosterol biosynthesis [14-16]. Therefore, these ergosterol-responsive promoters can be interesting candidates for the dynamic regulation of ERG9 expression in budding yeast.

Here, we investigated mRNA levels of ERG1, ERG11, ERG2 and ERG3 in an engineered yeast with relatively high mevalonate pathway activity [3]. Among these candidates, we found ERG11, ERG2 and ERG3 were transcriptionally down-regulated in our engineered strain when compared to the reference strain of BY4742, whereas mRNA level of ERG1 gene in both strains remained relatively low. When the engineered strains with ERG9 under the control of different ergosterol-responsive promoters were examined for the production of amorpha-4,11-diene (Figure 1), strains showed 2 ~ 5-fold higher levels of amorpha-4,11-diene than the reference strain. Among them, P_Erg1 showed the best result for improving amorpha-4,11-diene production yielding a final titer around 350 mg/L after 96 h cultivation in 250 mL shake-flasks. We envision dynamic control using side-product regulated systems could serve as an attractive strategy for redirecting metabolic flux towards product-of-interest. The methodology described here.

Figure 1 Schematic diagram of ergosterol-responsive promoters for controlling the metabolic flux towards ergosterol biosynthesis pathway. Genes from the mevalonate pathway in S. cerevisiae are shown in blue; heterologous expression of ADS gene is shown in green; and genes from ergosterol biosynthesis pathway are shown in red. The ERG9 expression is put under the control of ergosterol-responsive promoter (P_Erg) to achieve dynamic control of squalene synthase availability in response to intracellular ergosterol levels. The pathway intermediates IPP, DMAPP and FPP are defined as isopentenyl pyrophosphate, dimethylallyl pyrophosphate and farnesyl diphosphate, respectively.
would be generalizable for engineering other metabolic pathways.

**Results**

**Characterization of ERG1, ERG11, ERG2 and ERG3 expression levels in the engineered strains with high mevalonate pathway activity**

As ergosterol fulfills several essential functions that require optimal sterol concentrations, synthesis of sterols in yeast must be tightly regulated. For example, squalene epoxidase (encoded by ERG1) is an essential enzyme in the ergosterol-biosynthesis pathway and catalyzes the squalene epoxidation step. Inhibition of ergosterol biosynthesis with the antifungal drug terbinafine at squalene epoxidase step can trigger increased level of ERG1 expression in a concentration-dependent manner to a maximum of sevenfold [15]. Inhibition of a later step in the ergosterol biosynthetic pathway by ketoconazole, an inhibitor of the lanosterol-14α-demethylase (encoded by ERG11), also induces the expression of ERG1, indicating that ERG1 expression is positively regulated by diminished intracellular ergosterol levels. Similarly, various other ergosterol biosynthetic pathway genes such as ERG11 [17,18], ERG2 [16] and ERG3 [14] are transcriptionally up-regulated when ergosterol biosynthesis is inhibited using different drugs. Therefore, it is likely that ergosterol-responsive promoters – by definition – will respond to excessive amounts of intracellular ergosterol and trigger the down-regulation of corresponding genes accordingly.

In the present study, we decided to investigate whether the abovementioned genes, namely, ERG1, ERG11, ERG2 and ERG3, are transcriptionally downregulated in the engineered strain with relatively high mevalonate pathway activity. Previously, our group has successfully engineered yeast strains with significant improvement of mevalonate pathway activity for high-level production of amorpha-4,11-diene. Notably, we found that strain M4-2nd without ADS gene showed extremely slow growth expression levels between the reference strain BY4742, we found ERG11, ERG2 and ERG3 did show a sharp decrease of mRNA levels in the engineered strain M4-2nd over the reference strain BY4742, we found ERG11, ERG2 and ERG3 did show a sharp decrease of mRNA levels in the engineered strain M4-2nd (Figure 2). Intriguingly, there was no obvious change for ERG1 expression levels between the reference strain and the engineered strain (Figure 2), and the mRNA abundances of ERG1 in both strains were relatively low, which may also explain the accumulation of large amount of squalene in an engineered yeast with deregulated expression of HMG-CoA reductase, whereas the sterol contents showed only small changes [18].

**Amorpha-4,11-diene production in engineered strains with ERG9 under the control of different ergosterol-responsive promoters**

As different transcripts may have varying half-lives, the relative strengths of abovementioned ergosterol-responsive promoters are not reflected by qRT-PCR analysis as shown in Figure 2. The use of reporter genes such as lacZ under different ergosterol-responsive promoters may help determine the promoter strengths, but it will be too cumbersome to systematically evaluate the relative promoter strengths during different growth phases. Therefore, upon successful demonstration of transcriptional down-regulation of ergosterol biosynthesis genes in the engineered strain with high mevalonate pathway activity, we sought to directly test our engineered strains with ERG9 under the control of different ergosterol-responsive promoters for amorpha-4,11-diene production [20].

| Gene | Description |
|------|-------------|
| ERG10 | Acetyl-CoA C-acetyltransferase (EC:2.3.1.9) |
| ERG13 | Hydroxymethylglutaryl-CoA synthase (EC:2.3.3.10) |
| HMG1/2 | Hydroxymethylglutaryl-CoA reductase (EC:1.1.1.34) |
| ERG12 | Mevalonate kinase (EC:2.7.1.36) |
| ERG8 | Phosphomevalonate kinase (EC:2.7.4.2) |
| ERG19 | Diphosphomevalonate decarboxylase (EC:4.1.1.33) |
| IDI1 | Isopentenyl-diphosphate delta-isomerase (EC:5.3.3.2) |
| ERG20 | Bifunctional (2E,6E)-farnesyl diphosphate synthase/deglycerol kinase (EC:2.5.1.1.10) |

**Table 1 List of genes involved in the mevalonate and ergosterol biosynthesis pathways**

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In the present study, we decided to investigate whether the abovementioned genes, namely, ERG1, ERG11, ERG2 and ERG3, are transcriptionally downregulated in the engineered strain with relatively high mevalonate pathway activity. Previously, our group has successfully engineered yeast strains with significant improvement of mevalonate pathway activity for high-level production of amorpha-4,11-diene. Notably, we found that strain M4-2nd without ADS gene showed extremely slow growth expression levels between the reference strain BY4742, we found ERG11, ERG2 and ERG3 did show a sharp decrease of mRNA levels in the engineered strain M4-2nd over the reference strain BY4742, we found ERG11, ERG2 and ERG3 did show a sharp decrease of mRNA levels in the engineered strain M4-2nd (Figure 2). Intriguingly, there was no obvious change for ERG1 expression levels between the reference strain and the engineered strain (Figure 2), and the mRNA abundances of ERG1 in both strains were relatively low, which may also explain the accumulation of large amount of squalene in an engineered yeast with deregulated expression of HMG-CoA reductase, whereas the sterol contents showed only small changes [18].

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In the present study, all engineered strains with ERG9 under different ergosterol-responsive promoters were transformed with pRS415ADS to evaluate the effect of dynamic control of ERG9 expression on amorpha-4,11-diene production. The resulting strains were designated as M4-D1, M4-D2, M4-D3, and M4-D4 with the ERG1, ERG2, ERG3, and ERG11 promoters to drive ERG9 expression, respectively (Table 2). Interestingly, all engineered strains with ERG9 under different ergosterol-responsive promoters improved the amorpha-4,11-diene titer substantially, with up to a 5-fold improvement over the reference strain, which contains ERG9 under its native promoter (Figure 3B). Among these strains, strain M4-D1 showed the best result at 5-fold improvement and produced amorpha-4,11-diene to a final titer around 350 mg/L after 96 h cultivation in shake flasks, suggesting that ERG9 under the control of P_{ERG1} most efficiently restricted metabolic flux towards ergosterol biosynthesis. Moreover, there was an inverse correlation of amorpha-4,11-diene levels to growth rate during the early exponential phase (Figure 3A). As these ergosterol-responsive promoters can respond to the diminished level of ergosterol and compensate with elevated transcription levels [14,15,17], the perturbation of growth rate might be caused by the accumulation of toxic intermediates, as seen in E. coli [19].

As these ergosterol-responsive promoters can respond to the diminished level of ergosterol and compensate with elevated transcription levels [14,15,17], the perturbation of growth rate might be caused by the accumulation of toxic intermediates, as seen in E. coli [19]. To further confirm that the improved amorpha-4,11-diene titer in strains M4-D1 ~ D4 was attributed to the regulation of ERG9 expression, we next sought to systematically compare the mRNA abundance of ERG9 in all engineered strains. As can be seen from Figure 4, qRT-PCR analysis revealed that mRNA levels of ERG9 in strains M4-D1 ~ D4 were significantly lower when compared to the reference strain M4-2nd, indicating that the improvement of amorpha-4,11-diene levels was caused by ERG9 restriction using ergosterol-responsive promoters. Collectively, our findings suggested dynamic control of ERG9 expression using ergosterol-responsive promoters could be an alternative strategy for improving isoprenoid productions. Future work will be focusing on optimizing the dynamic-sensor device and FPP consumption module to allow the consumption of intermediates at the appropriate rates for optimal pathway activities.

**Discussion**

Dynamic regulation would allow an organism to adapt its metabolic flux to changes within the host or in its environment [21], which would allow the delivery of intermediates at the appropriate levels and rates for optimal pathway activities. In one of the pioneering examples of dynamic regulation system, acetyl phosphate was used as an indirect indicator for excess amount of glucolytic flux to regulate the heterologous lycopene biosynthesis pathway [22]. Recently, Zhang et al. demonstrated that dynamic regulation using FadR-based sensing device in E. coli could improve the FAEE production by 3-fold to 28% of theoretical maximum and also substantially improve the stability of biodiesel-producing strains [23]. In a more recent effort, Dahl et al. exploited the stress-response promoter system to dynamically control the mevalonate biosynthesis pathway in E. coli and the
resulting strain showed 2-fold improvement of amorpha-4,11-diene production compared to either a constitutive expression system or an IPTG inducible system [24]. Nature has provided abundant ligand-responsive transcription factors, whose DNA-binding activities are regulated by various types of molecules, including nucleic acids, carbohydrates, lipids, amino acids and many secondary metabolites [23]. For example, tyrR transcriptional factor from E. coli was reported to play an important role for regulating L-tyrosine biosynthesis pathway [25] and those tyrR-mediated tyrosine-responsive promoters may be used for dynamically controlling the side-pathway expressions and improving the product titer of tyrosine-derived compounds such as alkaloids.

High-level production of product-of-interest in microbial hosts also requires the metabolic flux towards competing pathways to be minimized or completely eliminated if it is not essential for the cells to survive [4-8]. However, many competing pathways inside the cell are producing compounds that are essential to support cell growth. For example, isoprenoid biosynthesis pathway is not only essential for ergosterol production to maintain proper membrane structure, but also for heme A and ubiquinone biosynthesis. Deletion of ERG9 gene to block metabolic flux towards biosynthesis of these essential components would result in inviable strains. Currently, researchers have explored the methionine-repressible MET3 promoter or copper-repressible CTR3 promoter for restricting ERG9 expression to divert metabolic flux from ergosterol biosynthesis to non-native isoprenoid production, which improved amorpha-4,11-diene production an additional 2-fold [9,10]. Other approaches such as harnessing a weak CYC1 promoter for controlling ERG9 expression or using a HXT1 promoter to couple ERG9 expression with glucose concentration also showed exciting results [4,11]. Here, we demonstrated that dynamic control of ERG9 expression using ergosterol feedback regulation mechanism could also substantially improve the amorpha-4,11-diene titer. As sterols fulfill several essential functions inside the cell, insufficient flux towards ergosterol and other sterols caused by restricting ERG9 expression was reported to cause deleterious effect on cell growth [6]. In this case, the amount and the timing of adding repressors such as copper sulfate and methionine to the broth during different growth phases was systematically investigated to avoid growth inhibition and to achieve the optimal product titer. In contrast, our dynamic control device harnesses ergosterol-responsive promoters to adjust ERG9 expression according to the cell’s need of ergosterols; the sterol level can be always maintained at an appropriate level for

| Table 2 List of plasmids and strains used in the present study |
|---------------------------------|---------------------------------|----------------|
| **Name** | **Description** | **References** |
| Plasmid name | | |
| pUC18 | Plasmid for cloning in E. coli | Invitrogen |
| pUG72 | Plasmid harboring URA3 selection marker | [30] |
| pSH68 | Plasmid harboring Cre gene under the control of P<sub>GAL1</sub> | [30] |
| pRS425ADS | prS425::P<sub>GAL1</sub>-ADS-T<sub>CYC1</sub> | [9] |
| pRS415ADS | pSH68 derivative with prS415::P<sub>GAL1</sub>-ADS-T<sub>CYC1</sub> | This study |
| pURA3-Blank | pJC18 derivative containing URA3 selection marker from pUG72 | This study |
| pURA3-ERG1p | pURA3-Blank derivative with insertion of promoter region from ERG1 gene | This study |
| pURA3-ERG11p | pURA3-Blank derivative with insertion of promoter region from ERG11 gene | This study |
| pURA3-ERG2p | pURA3-Blank derivative with insertion of promoter region from ERG2 gene | This study |
| pURA3-ERG3p | pURA3-Blank derivative with insertion of promoter region from ERG3 gene | This study |
| Strain name | | |
| M4-2nd | BY4742 derivative with the relatively high mevalonate pathway activity | [3] |
| M4-D1 | M4-2nd with ERG9 under the control of P<sub>ERG1</sub> | This study |
| M4-D2 | M4-2nd with ERG9 under the control of P<sub>ERG11</sub> | This study |
| M4-D3 | M4-2nd with ERG9 under the control of P<sub>ERG2</sub> | This study |
| M4-D4 | M4-2nd with ERG9 under the control of P<sub>ERG3</sub> | This study |
| M4-2nd-L | Strain M4-2nd harboring plasmid pRS415ADS | This study |
| M4-D1-L | Strain M4-D1 harboring plasmid pRS415ADS | This study |
| M4-D2-L | Strain M4-D2 harboring plasmid pRS415ADS | This study |
| M4-D3-L | Strain M4-D3 harboring plasmid pRS415ADS | This study |
| M4-D4-L | Strain M4-D4 harboring plasmid pRS415ADS | This study |

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Strains M4-D1 ~ D4 with ERG9 under the control of different ergosterol-responsive promoters were transformed with plasmid pRS415ADS for producing amorpha-4,11-diene. Here, strain M4-D1 ~ D4 are derived from strain M4-2nd with ERG9 under the control of P\textit{ERG1}, P\textit{ERG11}, P\textit{ERG2} and P\textit{ERG3}, respectively. Strain M4-2nd with plasmid pRS415ADS was used as control. Strains were cultivated in 250 mL flasks supplemented with 25 mL SC-LEU media. The amorpha-4,11-diene production in engineered strains was measured using GC-FID after 48 h and 96 h of cultivation. Values represented the average and standard deviation of three independent experiments.
optimal cell growth under changing environments (Figure 1). Once the cell senses the excess of ergosterol, ERG9 expression under the control of these ergosterol-responsive promoters will be tuned down, which will result in redirection of the metabolic flux towards non-native isoprenoid production. Another benefit of ergosterol-regulated system for dynamic control of ERG9 expression is that it also eliminates the requirement of adding repressors such as copper sulfate or methionine, which will significantly simplify the fermentation process, as well as reduce production costs.

In the future, promoter engineering using error-prone PCR can be further explored to improve the sensitivity and dynamic range of these ergosterol-responsive promoters, and to achieve even tighter control of gene expression. Alternatively, hybrid promoter systems may also be developed by fusion of ergosterol-responsive elements with other well-studied promoters. Furthermore, degradation signal through N-End rule [26,27] may be further used to modulate the enzyme turnover rate for a more robust and accurate dynamic control device.

**Conclusions**

In summary, a dynamic control device using side-product regulated system offers an alternative strategy to conventional approaches, such as gene deletion or CTR3/MET3 repressible promoters, for restricting metabolic flux towards side-product biosynthesis. The methodology described here would serve well as a generalized technique for engineering additional metabolic pathways.

**Methods**

**Strains, plasmids and reagents**

*Escherichia coli* strain DH5α was used for general plasmid constructions and the strain was cultivated at 37°C in Luria-Bertani medium with 100 μg/mL ampicillin. Previously engineered *S. cerevisiae* strain M4-2nd with high mevalonate pathway activity was used as the parental strain for all yeast strain constructions. This strain was cultured in rich YPD medium. Engineered strains with different auxotrophic selection markers were grown in synthetic complete (SC) medium with leucine or uracil dropout where appropriate. For induction of genes under the control of galactose inducible promoters, *S. cerevisiae* strains were grown in 1.8% galactose plus 0.2% glucose. Plasmid pSH68 and pUG72 were obtained from EUROSCRAFF. Plasmid pRS425ADS with the codon optimized amorpha-4,11-diene synthase (ADS) gene from the plant *Artemisia annua* [9] was kindly provided by Prof. Jay Keasling from University of California, Berkeley. Restriction enzymes, Taq polymerase, alkaline phosphatase (CIP) and T4 ligase were purchased from New England Biolabs (Beverly, MA). iProof HF polymerase and iScript™ Reverse Transcription Supermix were
obtained from BioRad (Hercules, CA). Gel extraction kit, PCR purification kit, Plasmid purification kit and RNaseasy Mini Kit were purchased from QIAGEN (Hilden, Germany). FastStart Essential DNA Green Master Mix was purchased from Roche (Singapore, SG). All of the chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO).

**Plasmid construction and yeast transformation**

Oligonucleotides used for plasmid construction are listed in Table 3. To create the genome integration cassette, a series of plasmids was constructed as follows. Firstly, *Kluyveromyces lactis* URA3 selection marker was amplified from genomic DNA of ERG9 cells was used for the total RNA extraction and quantitative real-time PCR.

Table 3 Oligonucleotides used for constructing plasmids and qPCR studies

| Name               | Description                                                                 |
|--------------------|-----------------------------------------------------------------------------|
| F_URA3_HindIII     | AGAGAAGACTGTCAGCGAGAACACGACCGACGGCCAAATACAACAGATACGCGT                      |
| R_URA3_BE          | GGATTCGGATCCAGGTGGTCTATCAGGGAGAAGAACGCG                                    |
| F_ERG1p_BamHI      | CGGGAATCGTCAACTACATATAGGCG                                                  |
| R_ERG1p_EcoRI      | GGATTCCTCAATTGTAATAGCTTTCCATGACCCTTTCTCCTAATGGT                             |
| F_ERG11p_BamHI     | CGGATCCCTGTTCTCTCTGCCTGTC                                                 |
| R_ERG11p_EcoRI     | GGATTCCTCAATTGTAATAGCTTTCCATGACCCTTTCTCCTAATGGT                             |
| F_ERG2p_BamHI      | CGGGAATCGTCAACTACATATAGGCG                                                  |
| R_ERG2p_EcoRI      | GGATTCCTCAATTGTAATAGCTTTCCATGACCCTTTCTCCTAATGGT                             |
| F_ERG3p_BamHI      | CGGGAATCGTCAACTACATATAGGCG                                                  |
| R_ERG3p_EcoRI      | GGATTCCTCAATTGTAATAGCTTTCCATGACCCTTTCTCCTAATGGT                             |
| F_URA3_BE          | GGATTCCTCAATTGTAATAGCTTTCCATGACCCTTTCTCCTAATGGT                             |
| R_ADS             | GGATTCCTCAATTGTAATAGCTTTCCATGACCCTTTCTCCTAATGGT                             |
| F_ACT1_q              | TCCGTCTGGATTGTTGJT             |
| R_ACT1_q              | TGGATCTTCCACATTTGTGGAAG         |
| F_ERG1_q            | TGGTGGTGCAAGGGTGTTG            |
| R_ERG1_q            | AATGTCAGTTGGGCTTGG             |
| F_ERG11_q          | TGCCGAGCCAAAACAAATTGGAAG       |
| R_ERG11_q          | CCTGGAGAAACAAACTG              |
| F_ERG2_q            | AATGTCGACTAGGCTGATTG          |
| R_ERG2_q            | TGAGAAAGTGGTCCAAAACC           |
| F_ERG3_q            | CAATACGGTCAATTCACCACTC         |
| R_ERG3_q            | AATGTCGACTAGGCTGATTG          |
| F_ERG9_q            | TTACATTTGGCGATCTGCC           |
| R_ERG9_q            | TTCCTGCAAAACACTCCAGTT          |

Amplified from plasmid pUG72 using primer pair F_URA3_HindIII/R_URA3_BE. The PCR product with size around 1.4 kb was purified, digested with HindIII/EcoRI and inserted into pUC18 cut with the same enzyme pair, to yield pURA3-Blank. Next, the 801 bp promoter region of ERG1 was amplified from genomic DNA of *S. cerevisiae* BY4742 using primer pair F_ERG1p_BamHI/R_ERG1p_EcoRI. The PCR product was cut with BamHI/EcoRI, and inserted into pUR3-Blank cut with BamHI/EcoRI, to yield pURA3-ERG1p. Similarly, the 1000 bp promoter region of ERG11, the 807 bp promoter region of ERG2, and the 802 bp promoter region of ERG3 were PCR amplified from genomic DNA of *S. cerevisiae*, cut with BamHI/EcoRI, and inserted into pUR3-Blank cut with BamHI/EcoRI, to yield plasmid pUR3-ERG11p, pUR3-ERG2p and pUR3-ERG3p, respectively (Table 2). To this end, these plasmids served as template for the amplification of genome integration cassette using primer pair F_ERG9p_Int/R_ERG9p_Int.

For generating the mutant strains with ERG9 under the control of different ergosterol-responsive promoters, electroporation was carried out as follows. Fresh overnight culture of strain M4-2nd was inoculated into 50 mL YPD medium to an initial OD600 of 0.3. Yeast cells were harvested by centrifugation at 4°C, 1500 g for 5 min after 4–5 h when OD600 reached 1.3. The cell pellet was washed twice with 50 mL ice-cold Milli-Q water, followed by centrifugation to collect the cells. Next, the cells were washed with 4 mL ice-cold 1 M sorbitol, pelleted by centrifuge and finally re-suspended in ice-cold sorbitol to a final volume of 400 μL. Subsequently, 50 μL of yeast cells together with approximately 2 μg of genome integration cassette were electroporated in a 0.2 cm cuvette at 1.6 kV. After electroporation, cells were immediately mixed with 2 mL pre-warmed YPD medium and shaken for 9 min on a rotary shaker to recover the cells. Cells were spotted on SC-URA plates and incubated at 30°C for 3–4 days until colonies appeared. Strains with successful replacement of native ERG9 promoter were verified by diagnostic PCR.

Since previous investigation showed that low copy expression of ADS gene resulted in higher amorpho-4,11-diene titers [28], centromeric plasmid based expression of ADS gene was created as follows. Briefly, ADS gene was amplified from pRS425ADS, cut with BamHI/XhoI and inserted into pSH68 cut with the same enzyme pair, to yield pRS415ADS. For the transformation of pRS415ADS into engineered strains, the standard lithium acetate method was used and the transformed cells were spotted on SC-LEU plates for selection.

**RNA extraction and quantitative real-time PCR**

Yeast cells were harvested at early-log phase and total amount of 1 × 107 cells was used for the total RNA extraction using the RNaseasy Mini Kit (QIAGEN, Germany).
Approximately 500 ng of RNA was converted to cDNA using iScript™ Reverse Transcription Supermix from BioRad (Hercules, CA).

The gene-specific primers for \textit{ERG9}, \textit{ERG1}, \textit{ERG11}, \textit{ERG2}, \textit{ERG3} and \textit{ACT1} were designed using the ProbeFinder (https://lifescience.roche.com), and oligonucleotides used for qRT-PCR experiment were listed in Table 3. Quantitative PCR analysis was carried out using LightCycler 96 real-time machine with FastStart Essential DNA Green Master Mix (Roche) according to the manufacturer's instructions. Each 20 μL reaction contained 50 ng of total cDNA, 10 μL FastStart Essential DNA Green Master Mix, 0.5 μM of each primer. Thermal cycling conditions were set as follows: pre-incubation, 1 cycle of 95°C for 10 min; amplification, 45 cycles of 95°C for 10 s, 57°C for 10 s and 72°C for 10 s. \textit{ACT1} was chosen as a reference housekeeping gene and the results were presented as ratios of gene expression between the \textit{ERG9}, \textit{ERG1}, \textit{ERG11}, \textit{ERG2}, \textit{ERG3} and the reference gene, \textit{ACT1} \cite{29}.

**Amorpha-4,11-diene production in engineered yeast**

To investigate the effect of ergosterol-responsive promoters on restricting \textit{ERG9} expression in the engineered strains, both the growth profile and amorpha-4,11-diene production profile were investigated. Strains harboring pRS415ADS were inoculated into SC-LEU medium. The next day, 250 mL flasks containing 25 mL SC-LEU medium (1.8% galactose + 0.2% glucose) were inoculated with fresh cell cultures to an initial OD$_{600}$ of 0.05. All flasks were immediately supplemented with 20% (vol/vol) dodecane after seeding, to perform two phase fermentation and harvest amorpha-4,11-diene. The growth profile was continuously monitored for 4 days. The amorpha-4,11-diene levels were measured after 48 h or 96 h cultivation. Every time, 100 μL of cell culture was taken for measuring OD$_{600}$ by microplate reader (Synergy H1, BioTek, USA), and 10 μL dodecane layer was sampled and diluted in 990 μL ethyl acetate for the quantitation of amorpha-4,11-diene levels using gas chromatography-flame ionization detector (GC-FID). For GC-FID analysis, 1 μL of diluted sample was injected into Shimadzu QP2010Ultra system equipped with a DB-5 column (Agilent Technologies, USA). Hydrogen was used as a carrier gas at a flow rate of 1.0 mL/min. The oven temperature was first kept constant at 80°C for 2 min, and then ramped to 190°C at a rate of 5°C/min, and finally increased to 300°C by 20°C/min. For the quantitation of amorpha-4,11-diene levels, caryophyllene was used for plotting the standard curve and the results shown in the present study are presented as caryophyllene equivalents.

**Competing interests**

The authors declare that they have no competing interests.

**Authors' contributions**

\textit{JY} conceived the project and designed the experiments. \textit{JY} performed the experiments and analyzed the data. \textit{JY} and \textit{CBC} wrote the manuscript. Both authors revised and approved the final manuscript.

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