Synthetic and Systems Biotechnology 10 (2025) 165–173

Contents lists available at ScienceDirect

Synthetic and Systems Biotechnology

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

Original Research Article

Systematic metabolic engineering of *Yarrowia lipolytica* for efficient production of phytohormone abscisic acid

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ARTICLE INFO

Keywords:
Abscisic acid
Phytohormone
Sesquiterpene
Yarrowia lipolytica
P450 enzymes

ABSTRACT

Abscisic acid (ABA) is an important phytohormone with diverse applications. It currently relies on the fermentation of *Botrytis cinerea*, which suffers from limited availability of genetic engineering tools. Here, *Yarrowia lipolytica* was engineered to enable *de novo* biosynthesis of ABA. To overcome the rate-limiting P450 enzymes, systematic engineering strategies were implemented. Firstly, the dissolved oxygen was increased to boost the activity of P450 enzymes. Secondly, the expansion of endoplasmic reticulum was implemented to improve the functional expression of P450 enzymes. Lastly, rate-limiting enzymes were assembled to facilitate substrate trafficking. Moreover, ABA production was further improved by strengthening the mevalonate pathway. Finally, the engineered strain produced 1221.45 mg/L of ABA in a 5-L bioreactor. The study provides effective approaches for alleviating rate-limiting P450 enzymes to enhance ABA production and achieve competitive industrial-level ABA production in *Y. lipolytica*.

1. Introduction

Abscisic acid (ABA), a sesquiterpenoid compound, was initially identified in plants during the 1960s [1]. As a pivotal phytohormone, ABA plays a crucial role in the agricultural sector, notably in regulating seed dormancy and cell elongation [2,3]. ABA also mediates adaptive responses to abiotic stress, such as drought or cold stress [4,5], and biotic stress, including pathogen infestation [6]. Moreover, studies have highlighted the potential health benefits of ABA supplementation. For instance, dietary intake of ABA-rich fruit extracts has been associated with lowered insulin and blood sugar concentrations in human [7]. Remarkably, elevated plasma ABA concentrations have been related to a decreased susceptibility to malaria infection [8].

ABA is widely distributed in higher plants and also in certain plant pathogenic fungi [9,10]. In plants, ABA is predominantly synthesized via the plastid methylerythritol phosphate (MEP) pathway, whereas in fungi, it follows the mevalonate pathway (MVA) [11]. Efforts to unveil the biosynthetic pathway of ABA have been driven by its diverse functionality. In *Botrytis cinerea*, ABA synthesis initiates with farnesyl diphosphate (FPP). FPP is subjected to cyclization via α-ionylideneethane synthase (*Bc*Aba3), resulting in α-ionylideneethane. Then, two P450 enzymes (*Bc*Aba2 and *Bc*Aba2) facilitates the oxygenation of α-ionylideneethane to form α-ionylideneacetic acid and 1′, 4′-trans-dihydroxy-α-ionylideneacetic acid, respectively. Ultimately, a dehydrogenase (*Bc*Aba4) catalyzes this compound to form ABA [12].

At present, ABA is industrially produced using the native fungus *B. cinerea* [13]. However, the process involves significant costs as a result of extensive separation procedures and extended fermentation durations. Furthermore, *B. cinerea* has limited genetic engineering tools available, posing significant challenges for metabolic engineering endeavors. Consequently, extensive exploration has been undertaken to establish ABA biosynthesis to heterologous hosts with more accessible molecular tools. Reconstructing the ABA biosynthesis pathway from *B. cinerea* has facilitated ABA production in heterologous microbes such as *Aspergillus oryzae* (8 mg/L) [12], *Saccharomyces cerevisiae* (11.4 mg/L) [14], and *Y. lipolytica* (263.5 mg/L) [15]. Recently, the production of ABA has a notable improvement compared with previous reports. Liu et al. engineered *Y. lipolytica* for overproducing ABA and achieved a final titer of 1.21 g/L by optimizing the MVA pathway, exogenous ABA synthesis pathway, mitochondrial localization strategy, and adding

Peer review under responsibility of KeAi Communications Co., Ltd.
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https://doi.org/10.1016/j.synbio.2024.10.004
Received 6 July 2024; Received in revised form 14 September 2024; Accepted 17 October 2024
Available online 18 October 2024

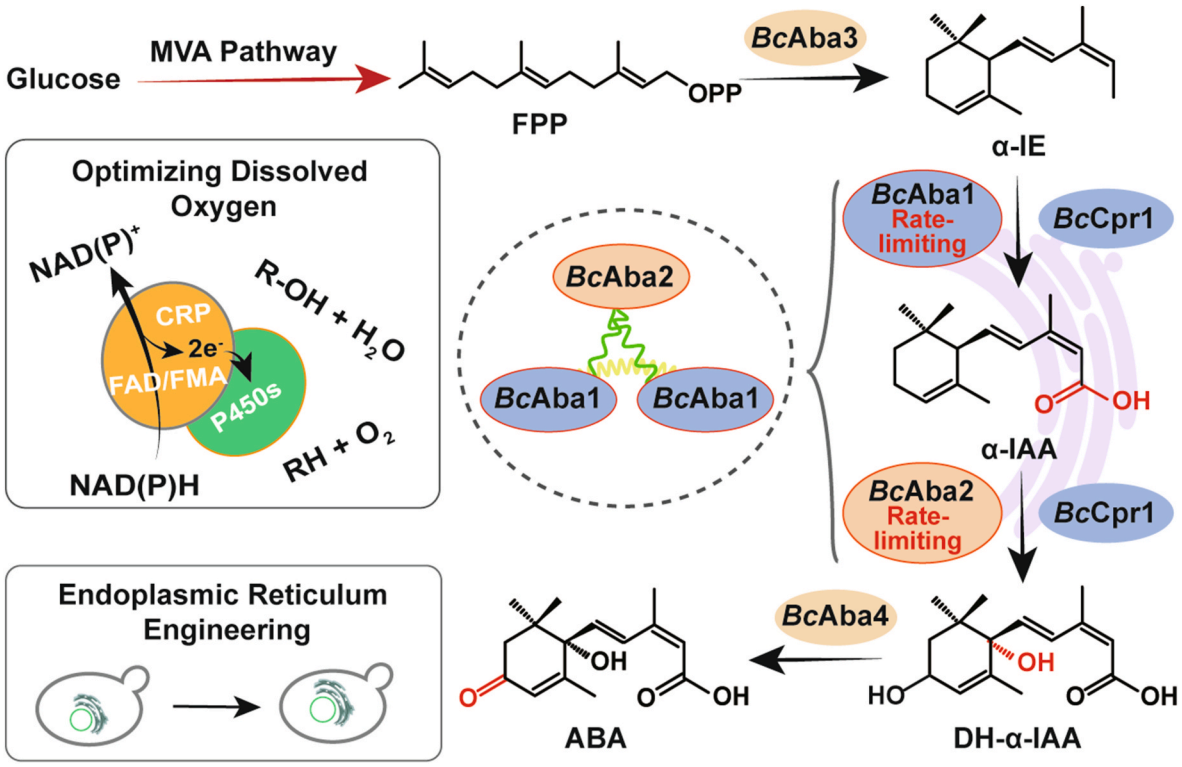


Fig. 1. Schematic representation of the ABA biosynthesis pathway and summary of the metabolic strategies used in this study. *Bc*Aba3, *B. cinerea* α-ionylideneethane synthases; *Bc*Aba1, *B. cinerea* cytochrome P450; *Bc*Aba2, *B. cinerea* cytochrome P450; *Bc*Aba4, *B. cinerea* dehydrogenase; *Bc*Cpr1, *B. cinerea* cytochrome P450 reductase. FPP, farnesyl pyrophosphate; α-IE, α-ionylideneethane; α-IAA, α-ionylideneacetic acid; DH-α-IAA, 1′,4′-trans-dihydroxy-α-ionylideneacetic acid; ABA, abscisic acid.

dodecane during fed-batch fermentation [16].

The biosynthetic pathway of ABA involves two P450 enzymes, *Bc*Aba1 and *Bc*Aba2, which were identified as the rate-limiting steps [15]. Low activity or poor expression of P450 enzymes is a widely encountered challenge in biotechnology and synthetic biology. However, previous ABA production focused mainly on engineering the isoprenoid biosynthetic pathway and redox cofactor NADPH, no studies have focused on improving the expression and activity of P450 enzymes in the ABA biosynthesis pathway. Engineering P450 enzymes is considered a major challenge [17], and manipulating two P450s in series within heterologous microbes presents a significant obstacle in ABA biosynthesis.

The oleaginous yeast *Yarrowia lipolytica* is considered as a superior chassis cell for heterologous expression of P450s for terpenoid modification, which also has high concentration of acetyl-CoA [17,18]. In this study, *Y. lipolytica* was tailored as an alternative host for ABA production. In order to enhance the heterologous expression of P450s in ABA pathway, systematic metabolic engineering was implemented, including increasing dissolved oxygen, engineering the endoplasmic reticulum, and assembling rate-limiting P450 enzymes with short peptide tags (RIAD and RIDD) (Fig. 1). Furthermore, the efficient conversion of acetyl-CoA to the downstream isoprenoid synthesis often acts as a bottleneck for terpene production in microbial chassis cells [19]. The established *Y. lipolytica* platform focused on overexpressing critical enzymes of the MVA pathway [15,16], while neglecting the manipulation of gene expression across the entire pathway. In this study, the complete MVA pathway was integrated into *Y. lipolytica* to enhance the synergistic effect of the pathway and drive the metabolic flux to the MVA pathway. The yield of ABA was gradually improved. Ultimately, *Y. lipolytica* was engineered to achieve a gram-scale ABA titer (1221.45 mg/L). This study not only improved abscisic acid production in yeast but also provided a generic method for manipulating multiple P450 enzymes in microbes.

2. Materials and methods

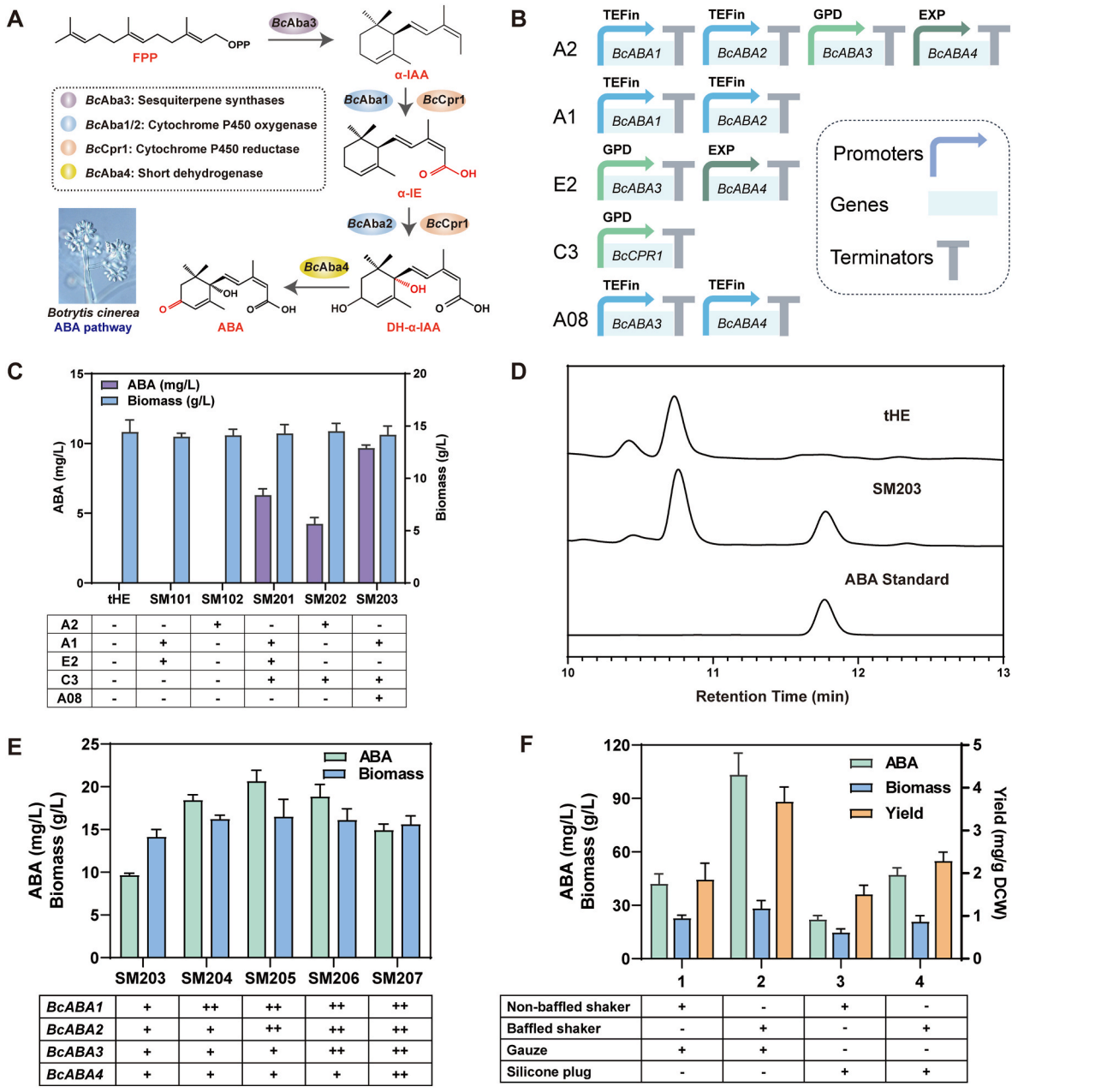
2.1. Strains and media

The yeast strains were derived from *Y. lipolytica* Polf (ATCC MYA-2613) (Supplementary Table 1). *Y. lipolytica* Polf-Δku70 was derived from Polf by disrupting the KU70 gene to enhance homologous recombination efficiency [20]. Yeast extract peptone dextrose (YPD) medium (1 % yeast extract, 2 % tryptone, and 2 % glucose) was used for strain growth. Synthetic complete medium devoid of uracil (SC-Ura) or leucine (SC-Leu) was used for selecting strains harboring URA3-or LEU2-based plasmids, which contain 2 % glucose, 0.67 % yeast nitrogen base without amino acids, 0.077 % amino acid mix lacking Ura or Leu, and 2 % agar. 5-fluoroorotic acid (5-FOA) plates (YPD plate + 0.8 g/L 5-FOA) were used to remove the URA3 marker. Shake-flask fermentation was conducted in 250 mL non-baffled or baffled flasks with 50 mL YPD80 medium (1 % yeast extract, 2 % tryptone, and 8 % glucose). The strains were cultured at 30 °C with continuous shaking at 200 rpm for 120 h, starting with an initial inoculation OD_{600} of 0.5.

Escherichia coli DH5α was applied to the construction of plasmids. The recombinant *E. coli* strains were cultivated at 37 °C in Lysogeny Broth supplemented 0.1 % ampicillin.

2.2. Plasmids and strain construction

The codon-optimized genes were synthesized by GenScript (Nanjing, China) (Supplementary Table 4). The endogenous genes, promoters and terminators were PCR amplified using Phanta Super Fidelity DNA Polymerase (Vazyme, Nanjing, China), with the genome of *Y. lipolytica* Polf-Δku70 as the template. All gene fragments were obtained via PCR amplification using specific primers (Supplementary Table 3). PCR fragments were isolated using the DNA Fragment Purification Kit (Takara, Dalian, China), and the recombinant plasmids were prepared



	tHE	SM101	SM102	SM201	SM202	SM203
A2	-	-	+	-	+	-
A1	-	+	-	+	-	+
E2	-	+	-	+	-	-
C3	-	-	-	+	+	+
A08	-	-	-	-	-	+

	SM203	SM204	SM205	SM206	SM207
BcABA1	+	++	++	++	++
BcABA2	+	+	++	++	++
BcABA3	+	+	+	++	++
BcABA4	+	+	+	+	++

	1	2	3	4
Non-baffled shaker	+	-	+	-
Baffled shaker	-	+	-	+
Gauze	+	+	-	-
Silicone plug	-	-	+	+

Fig. 2. Reconstruction and optimization of abscisic acid pathway in *Y. lipolytica*. (A) Abscisic acid gene cluster, responsible for ABA biosynthesis, originates from the *Botrytis cinerea*. (B) Different gene configuration of abscisic acid gene cluster. (C) ABA production by strains expressing ABA gene cluster in different configuration. (D) Results of HPLC chromatograms of the engineered strain SM203, the control strain tHE and authentic ABA standard. (E) The effect of a second abscisic acid gene overexpression on the titer of ABA. (F) The effect of dissolved oxygen concentration on the titer of ABA.

using the ClonExpress MultiS One Step Cloning Kit (Vazyme, Nanjing, China) (Supplementary Table 2). The methods for strain construction and positive clones verification were described previously [21].

2.3. Sample preparation for abscisic acid analysis

To quantify the intracellular ABA concentration, 1 mL of culture broth was centrifugated at 10, 000 g for 5 min, and the cell pellets were resuspended in water. This washing process was repeated. Subsequently, 1 mL of methanol and 0.5 g of glass beads were added. The mixture underwent thorough dispersion by employing a homogenizer through ten cycles, each lasting 10 s at 5500 rpm. Subsequently, centrifugation at 10000*g* for 5 min was conducted. Finally, the supernatant methanol was used for HPLC analysis.

To quantify the extracellular ABA concentration, 1 mL of the culture broth was centrifuged at 10000 g for 5 min, and the resulting supernatant was used for product quantification. In certain instances, the culture broth should be diluted prior to analysis.

2.4. Product quantification

The samples were analyzed using high-performance liquid chromatography (HPLC) (Agilent 1260 Infinity II, USA) with an ultraviolet detector (Agilent 1260 VWD, USA). The HPLC detection were conducted with C18 column (4.6 × 150 mm, 4 μm) (Agilent Poroshell 120, USA). The injection volume is 5 μL and the temperature was maintained at 30 °C. The eluent was 70 % methanol and the pH is adjusted to 3.0 with phosphoric acid. Flow was set at 0.4 mL/min. The ABA concentrations

were calculated from authentic calibration standards. Biomass and the concentration of glucose was measured as described before [21].

2.5. Fluorescence detection

A single colony was picked in 5 mL of YPD medium (200 rpm, 24 h). Afterward, red and green fluorescence were examined using confocal laser scanning microscopy (Olympus FV3000, Japan). The excitation wavelengths were 485 nm (sfGFP) and 588 nm (mCherry).

2.6. Fed-batch fermentation

A single colony was picked to 5 mL YPD, then shaken at 200 rpm for 20–24 h until the OD_{600} reached approximately 5.0. The primary culture was inoculated in 500 mL non-baffled flasks with 100 mL YPD to initial inoculation OD_{600} of 0.5. Incubation of the shake flask to achieve the second seed culture with an OD_{600} of around 10.0.

The high-density fermentation was conducted in 5-L bioreactor (T&J Bioengineering Co., Ltd, Shanghai, China) with an initial volume of 3 L. The second seed cultures were injected into the bioreactor to reach initial OD_{600} of 0.5. The culture medium contained 150 g/L glucose, 6 g/L yeast extract, 12 g/L ammonium sulfate, 1.5 g/L $MgSO_4{\cdot}7H_2O$, 6 g/L KH_2PO_4, 3 g/L $Na_2HPO_4{\cdot}12H_2O$, 3 mg/L $CaCl_2{\bullet}2H_2O$, 1 mL/L trace metals stock (1000 ×), and 1 mL/L vitamins stock (1000 ×) [22]. The pH was controlled at 5.5 by automatically adding 10 M NaOH. The stirring speed was automatically adjusted between 300 and 1000 rpm and the air input was controlled at 6 vol per volume per minute (vvm) to maintain the dissolved oxygen (DO) at 30 %. The feeding glucose and ammonium sulfate with a C/N ratio of 30:1 was continuously pumped to meet consumption after 48 h. Samples were collected every 12 h to assess glucose concentration, dry cell weight (DCW), and ABA composition.

2.7. Statistical analysis

The results were expressed as the mean ± standard deviation for 3 biological replicates. All statistical evaluations (*p*-values) were performed using a two-tailed *t*-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3. Results and discussion

3.1. Engineering Yarrowia lipolytica for de novo biosynthesis of abscisic acid

The biosynthesis of ABA involves a gene cluster consisting of four biosynthetic genes, denoted as *BcABA1-BcABA4*, derived from *B. cinerea*, which catalyze a sequential four-step enzymatic process (Fig. 2A). Previously, *Y. lipolytica* was engineered for enhanced flux of MVA pathway and this corresponding strain, tHE, served as the starting strain for this work [23]. The modifications included the overexpression of truncated 3-hydroxy-3-methylglutaryl coenzyme A reductase (tHmg1) and farnesyl diphosphate synthase (Erg20). In order to construct the complete ABA biosynthesis pathway, the four biosynthetic genes, *BcABA1- BcABA4* were integrated into the tHE strain. Different genetic constructs, varying in gene order and configuration, can impact the gene expression level [24]. Thus, the four genes were assembled in two configurations to explore the effects of different assembly modes of the multi-step heterologous pathway (Fig. 2B). The two assembly modes are: (A) *BcABA1* and *BcABA2* were integrated into the IntA site, *BcABA3* and *BcABA4* were integrated into the IntE2 site to generate the strains SM101; (B) *BcABA1*, *BcABA2*, *BcABA3* and *BcABA4* were integrated in the IntA site to generate the strains SM102. However, no ABA could be observed in the two resultant strains.

It was speculated that the native *Y. lipolytica* cytochrome P450 reductase (CPR) might be incompatible with heterologous cytochrome P450 enzymes (P450s, CYP) in ABA biosynthesis pathway. Therefore, the corresponding *B. cinerea* CPR gene, referred to as *BcCPR1*, was co-expressed in SM101 and SM102 to generate the strain SM201 and SM202, capable of producing detectable levels of ABA. Notably, strain SM201 produced 6.29 mg/L ABA, marking a 48.7 % increase compared to SM202 (4.23 mg/L) (Fig. 2C). There may be a transcriptional interference of multiple heterologous genes when the four genes were expressed in the same site in strain SM202 [24]. This also indicates co-expression of heterologous CYPs and their redox partners CPRs is highly significant to ensure maximal catalytic efficiency of heterologous CYPs.

Promoters are crucial elements that regulate gene expression levels. Generally, increasing promoter strength is a common method to enhance the transcriptional level of a target gene [25]. The TEF promoter (from the translation elongation factor 1-α gene) is a constitutive, strong promoter in *Y. lipolytica*, and when an 113bp intron is added to the TEF promoter, the resulting TEFin promoter is 17 times more potent than the TEF promoter [26]. Replacing the promoter in the *BcABA3* and *BcABA4* expression cassette with the strong TEFin promoter in SM201 led to a significant increase in ABA levels, from 6.29 mg/L to 9.67 mg/L (SM203, Fig. 2D). The results indicate that strong promoters are conducive to balancing the metabolic flux of the multi-step heterologous ABA biosynthesis pathway, thereby enhancing the synthesis of ABA in *Y. lipolytica*.

3.2. Identifying rate-limiting steps via multi-copy strategy

For multigenic pathway engineering, metabolic flux imbalances are a widely encountered challenge due to the accumulation of metabolic intermediates caused by the different expression strength of multiple genes in the pathway. The absence of commercial reference standards for intermediate metabolites has posed challenges in identifying metabolic bottlenecks in ABA pathway. Therefore, all the enzymes in ABA biosynthesis pathway were overexpressed in strain SM203 to explore the bottleneck, resulting in strains SM204-SM207 (Fig. 2E). The strain SM204 (*BcABA1*) and SM205 (*BcABA1* and *BcABA2*) produced 19.80 and 24.60 mg/L ABA, respectively, representing a 2.05 and 2.54-fold increase in ABA titers compared to SM203. The overexpression of *BcABA3* and *BcABA4* in strain SM205 was continued to generate strain SM206 and SM207. Nevertheless, the concurrent expression of these genes led to a reduced ABA concentration compared to SM205 (Fig. 2E). It was speculated the expression of *BcABA3* and *BcABA4* caused the accumulation of intermediate that cannot be converted to ABA efficiently, which may have feedback inhibition on the enzyme. Consequently, the two P450 enzymes were considered the primary bottleneck in the ABA biosynthetic pathway, aligning with findings from prior studies [14,15].

In aerobically engineered microorganisms, the dissolved oxygen concentration is an important factor for the productivity during the fermentation process. In addition, *Bc*ABA1 and *Bc*ABA2 belong to the P450 enzyme family, which uses oxygen and the cofactor NAD(P)H (mediated by redox chaperones) to facilitate the incorporation of oxygen atoms into C–H bonds (Fig. 1) [18,27]. The hydroxylation reaction catalyzed by P450 enzymes requires oxygen, therefore, improving the oxygen supply is an important way to improve the efficiency of aerobic reactions and cell growth [17]. To evaluate the effect of oxygen availability on ABA production, different types of 250 mL shake flasks (non-baffled or baffled shaker) and bottle plug (silicone plugs and gauze) were used to cultivate SM205 for ABA production (Supplementary Fig. 1). As shown in Fig. 2F, the titer of ABA varies greatly under different culture conditions. The highest titer of ABA (103.23 mg/L) was achieved in 250 mL baffled flasks with gauze as the bottle plug. *Y. lipolytica* is a strict aerobic microorganism, and the increased oxygen contributes to better growth. The biomass increases from 14.70 g/L to 28.30 g/L. Therefore, specific titers, expressed as mg/g DCW rather than mg/L, are used for strain evaluation, with the latter being less sensitive to variations in biomass. The highest specific

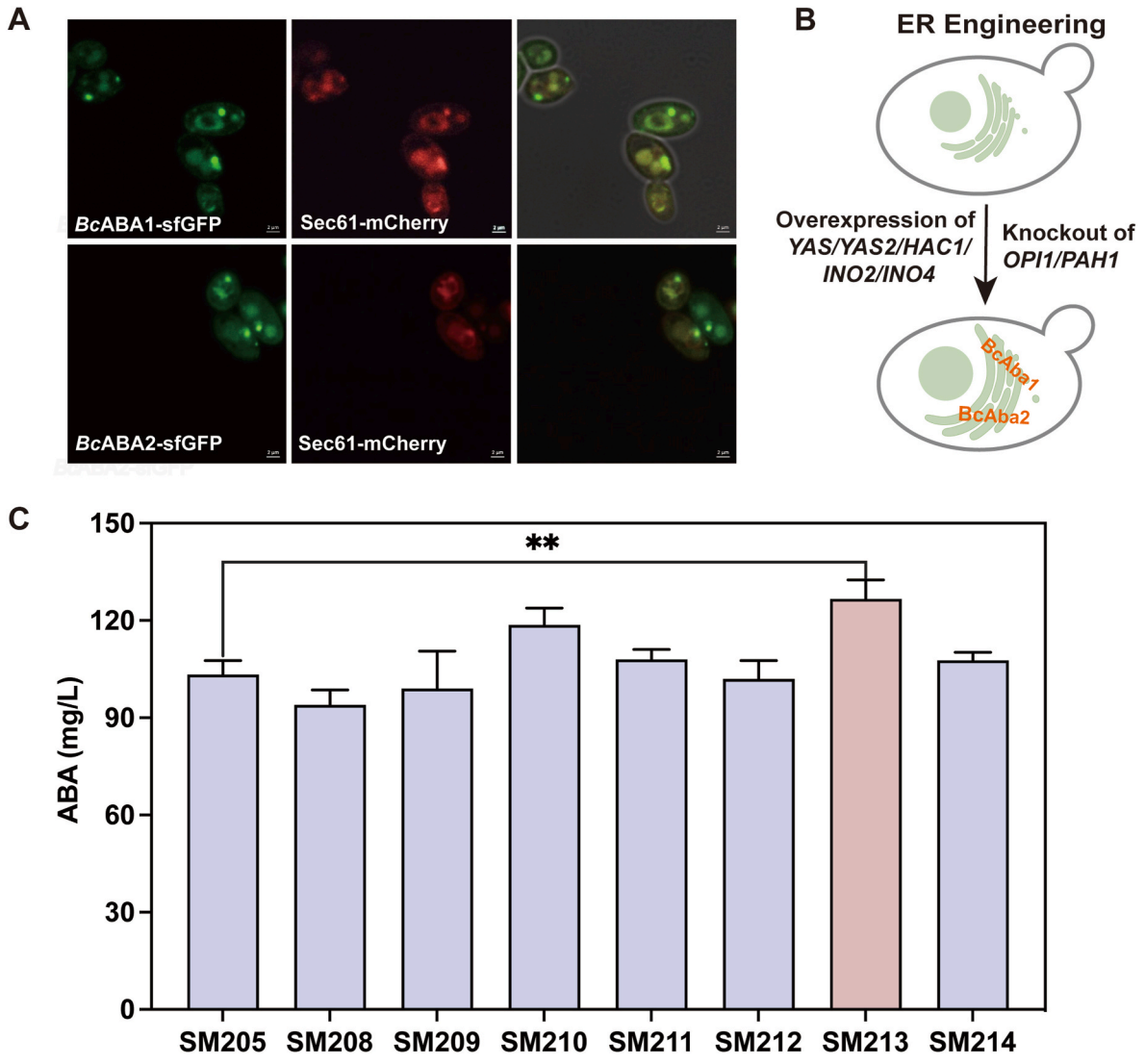


Fig. 3. The effects of abscisic acid production by endoplasmic reticulum engineering. (A) Visualized analysis of VA-01 and VA-02. The first row are *Bc*Aba1-sfGFP, Sec61-mCherry and merge image. The second row are *Bc*Aba2-sfGFP, Sec61-mCherry and merge image. (B) Illustration of different engineering targets (individual overexpression of *YAS1/YAS2/HAC1/INO2/INO4* and knockout of *OPI1/PAH1* for rewiring ER size to accommodate additional protein-folding loads. (C) Production of ABA in the strains with different engineering targets. The data is reported as the mean ± standard deviation from three distinct biological replicates. The statistical significance (p-value) was performed by a two-tailed *t*-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

titer (3.68 mg/g DCW) was obtained in 250 mL baffled flasks with gauze as the bottle plug. This suggests that the increased oxygen supply not only promotes cell growth but also boosts the efficiency of the ABA pathway in *Y. lipolytica*.

3.3. Rewiring endoplasmic reticulum size to enhance P450-mediated metabolic conversion

Most P450 enzymes are naturally situated in the endoplasmic reticulum (ER), with a few residing in mitochondria [28]. As membrane-bound proteins, confirming the subcellular localization of P450 enzymes in heterologous systems is crucial. This information can guide rational engineering efforts to enhance the expression and catalytic efficiency of these proteins. The prediction results of subcellular localization for *Bc*Aba1 and *Bc*Aba2 indicate their localization within the ER (Supplementary Fig. 1). To confirm the actual sublocation in *Y. lipolytica*, *Bc*Aba1 and *Bc*Aba2 were individually fused with sfGFP. Concurrently, the ER marker protein Sec61 was linked with mCherry. *Bc*Aba1-sfGFP and *Bc*Aba2-sfGFP were co-expressed with Sec61-mCherry, respectively, resulting in strain VA-01 and VA-02. Fluorescence microscopy revealed the colocalization of *Bc*Aba1-sfGFP and *Bc*Aba2-sfGFP with Sec61-mCherry (Fig. 3A). These findings indicate that the two P450 enzymes, *Bc*Aba1 and *Bc*Aba2, were localized to the ER, in accordance with the in silico results in native organisms.

ER is a dynamic organelle and its dramatic expansion will facilitate the functional expression of ER-localized proteins. Furthermore, increasing the ER volume enhances the capacity for protein synthesis and folding within the ER, while simultaneously alleviating metabolic constraints caused by enzyme deficiencies [29]. Consequently, efforts are directed towards engineering *Y. lipolytica* to expand the ER, thereby increasing the probability of active expression and correct folding of P450s enzymes. The ER is regulated by numerous factors [28,30]. Notably, phospholipids constitute the predominant constituent of the ER membrane, the comprehensive regulation of phospholipid synthesis involves transcription factors such as *INO2*, *INO4*, and *OPI1* [30]. Boosting the expression of *INO2* or *INO4* (which encode a transcription factor complex essential for controlling ER size) and deleting *OPI1* gene (which encodes a suppressor for the *INO2/INO4*) are frequently targeted modifications for expanding the ER. The *INO2* and *INO4* complex has been extensively studied for improving the production of terpenoids in

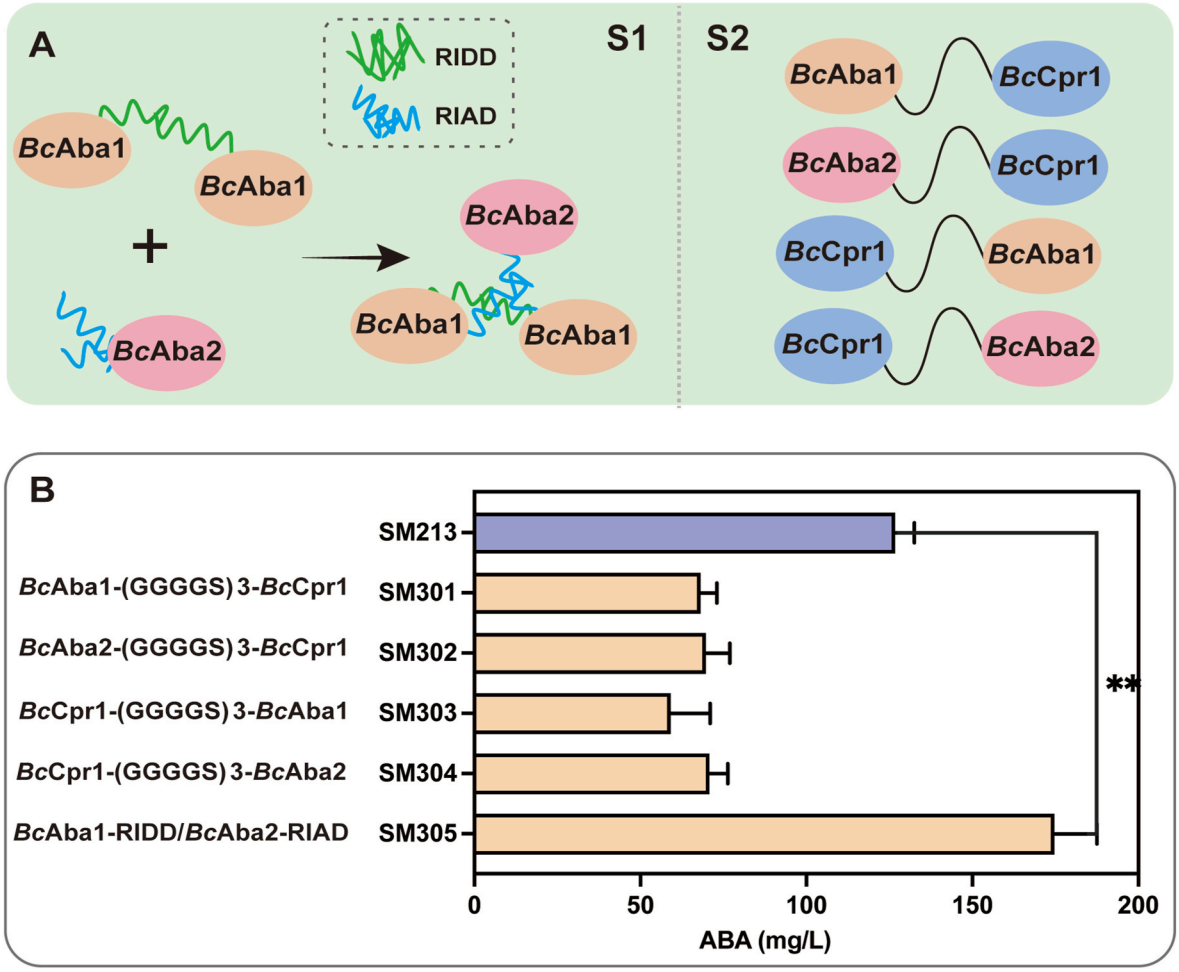


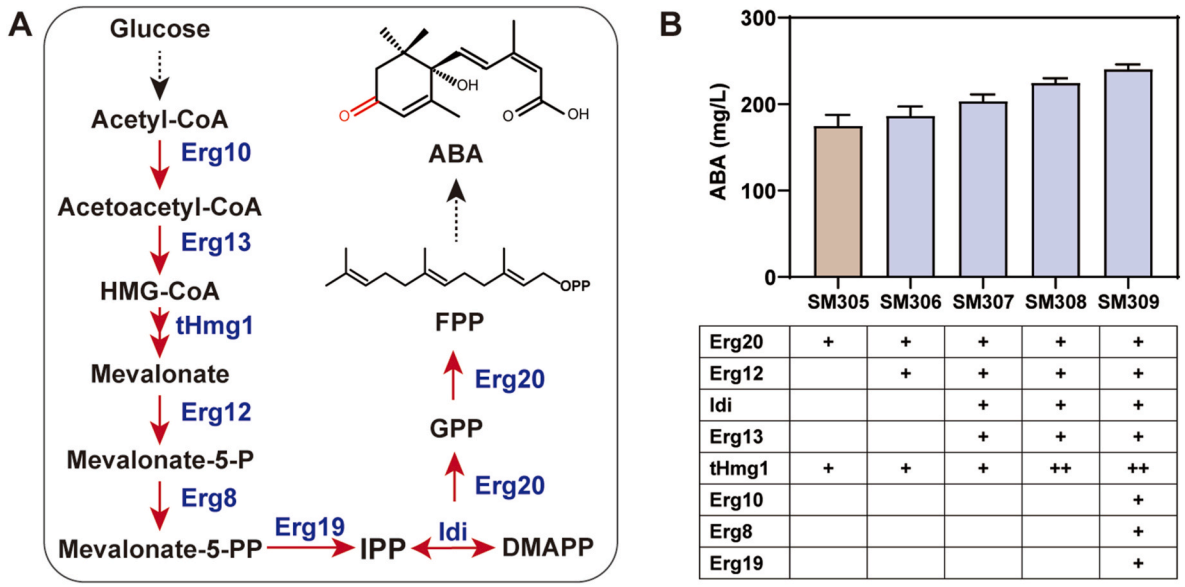
Fig. 4. Effects of different assembly strategies of rate-limiting enzyme on the titers of abscisic acid. (A) Illustration of strategies for facilitating substrate trafficking by assembling rate-limiting enzymes in ABA biosynthesis pathway. S1 (Strategy1), rate-limiting enzymes fused with RIAD and RIDD. S2 (Strategy2), CYPs and CPRs fused by $GGGGS_3$. (B) Changes of ABA titer by assembling rate-limiting enzymes. The data is reported as the mean ± standard deviation from three distinct biological replicates. The statistical significance (p-value) was performed by a two-tailed *t*-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

S. cerevisiae. Overexpression of *YlINO2* resulted in ER membrane expansion and improved lipid production in *Y. lipolytica* [22]. Nevertheless, the impact of overexpressing *INO2/INO4* on terpenoid engineering in *Y. lipolytica* has yet to be investigated. Additionally, *YAS1/YAS2*, has been identified to share some similarity with *INO4/INO2*, which upregulates genes involved in phospholipid synthesis [31]. The disruption of *PAH1*, encoding phosphatidic acid phosphatase, is a common strategy for expanding both the outer nuclear membrane and the ER, a phenomenon termed ER proliferation [32]. *HAC1* acts as a transcription factor, regulating the unfolded protein response triggered by ER stress [22]. Due to the complex regulation of the organelle by numerous factors, manipulation of different targets had varying effects on product synthesis. For example, disrupting the *PAH1* gene in *S. cerevisiae* resulted in an 8-fold increase in the accumulation of triterpenoid, β-amyrin, a 6-fold increase in medicagenic acid, and a 16-fold increase in saponins [32]. Although overexpressing *INO4* and deleting *OPI1* did not enhance squalene and protopanaxadiol production, overexpression of *INO2* improved squalene and protopanaxadiol yields significantly, by 71-fold and 8-fold respectively [30]. The disruption of *OPI1* significantly enhanced the yield of costunolide, whereas the production of parthenolide was enhanced by HAC1 overexpression [28].

Consequently, SM205 was engineered to increase the size of the ER by overexpression of *INO2*, *INO4*, *YAS1*, *YAS2* and *HAC1* and disruption of *OPI1* and *PAH1*, with the aim of reducing ER stress (Fig. 3B). As demonstrated in Fig. 3C, both the deletion of *PAH1* and the overexpression of *HAC1* improved ABA production, with the knockout of *PAH1* achieving the highest titer of 126.67 mg/L (SM213). Conversely, the overexpression of *YAS1* or *YAS2* led to a reduction in ABA production, potentially attributed to the fact that *YAS1/YAS2* exclusively upregulates CYP genes in response to alkanes but not other P450 enzymes in the ABA biosynthetic pathway [31]. *INO2/INO4* and *OPI1* are key transcriptional regulators of phospholipid biosynthetic genes [28]. In addition to their role in regulating the ER, they also function as global regulators of lipid metabolism. Recent studies have shown that overexpression of *INO2/INO4* upregulates genes involved in lipid biosynthesis, which shares acetyl-CoA as a common precursor with ABA synthesis [29]. Disruption of *PAH1* not only leads to a significant expansion of the ER but also positively influences ER-associated P450 enzymes involved in sesquiterpenoid biosynthesis, such as artemisinic acid [32]. This may explain why *PAH1* knockout enhances ABA production.

3.4. Facilitating substrate trafficking by assembling rate-limiting enzymes

Facilitating substrate trafficking within synthetic pathways is crucial for optimizing product yield through local confinement of the rate-limiting enzymes. Modular enzyme assembly approaches have been used to regulate these concentrations. This involves linking rate-limiting enzymes through either a short protein linker [33] or a peptide pair



Erg20	+	+	+	+	+
Erg12		+	+	+	+
Idi			+	+	+
Erg13			+	+	+
tHmg1	+	+	+	++	++
Erg10					+
Erg8					+
Erg19					+

Fig. 5. Enhancing the flux of the mevalonate pathway. (A) Schematic illustration of the MVA pathway leading to the production of ABA. (B) Boosting ABA production by fully utilizing the MVA pool. IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP: geranyl diphosphate; FPP, farnesyl pyrophosphate. The data is reported as the mean ± standard deviation from three distinct biological replicates. The statistical significance (p-value) was performed by a two-tailed *t*-test (*p < 0.05, **p < 0.01, ***p < 0.001).

(Fig. 4A) [34]. These fusion closely mimic natural metabolic regulation mechanisms, facilitating the activation of enzyme cascades with elevated local concentrations of intermediates through enhanced enzyme proximity [35]. Notably, in the case of P450 enzymes, the fusion of CYP with CPR using a short protein linker has proven successful in facilitating electron delivery and improving the enzyme catalysis [36, 37]. Additionally, the construction of multi-enzyme complexes, based on short peptide tags (RIAD and RIDD), has also been used to optimize the synthesis of terpenoids. The specific binding of the RIAD peptide to the RIDD dimer guides the fused enzymes to adopt a 2:1 binding stoichiometry, resulting in higher catalytic efficiency compared to enzyme fusion with a linker in some cases [33,38].

To relieve the bottlenecks in ABA biosynthesis pathway, the P450 enzymes were fused through a short protein linker or a pair of short peptide tags described above. With the aim to improve electron transfer efficiency, the rate-limiting enzymes *Bc*Aba1 and *Bc*Aba2 were fused to *Bc*Cpr1 with different enzyme orientation via a short protein linker ($GGGGS_3$). Here, four strains were constructed: SM301 (*Bc*Aba1-$GGGGS_3$- *Bc*Cpr1), SM302 (*Bc*Aba2-$GGGGS_3$- *Bc*Cpr1), SM303 (*Bc*Cpr1-$GGGGS_3$- *Bc*Aba1), SM304 (*Bc*Cpr1-$GGGGS_3$-Bcaba2). The RIDD or RIAD were attached to the C-terminus of *Bc*Aba1 and *Bc*Aba2, resulting in the strain SM305 (*Bc*Aba1-RIDD/*Bc*Aba2-RIAD). As shown in Fig. 4B, strain SM305 yielded 175.34 mg/L of ABA, marking a 38.4 % increase compared to the control strain SM213. The improved production of ABA in SM305 can be attributed to two factors. First, the assembly of *Bc*Aba1 and *Bc*Aba2 using RIDD/RIAD shortens the spatial distance between the rate-limiting enzymes, creating substrate channeling that improve abscisic acid synthesis. Second, the increased copies of *Bc*Aba1 with RIDD tags efficiently release the rate-limiting step. However, the direct fusion of rate-limiting enzymes results in a decrease of their functionality. One possible reason for this could be that the active site is located in the N- or C-terminal region, causing enzyme inactivation due to fusion expression [33]. Additionally, fusion of large enzyme may experience a reduction of the activity, potentially leading to complete loss of function [39].

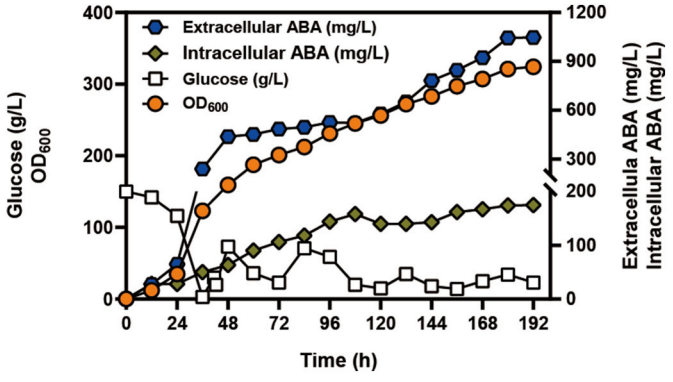


Fig. 6. Abscisic acid production by strain SM309 in 5-L fed-batch fermentation. Quantification of extracellular and intracellular ABA, glucose and biomass was conducted every 12h.

3.5. *Improving abscisic acid production by strengthening the mevalonate pathway*

As a 15-carbon terpene derivative, ABA is synthesized by the MVA pathway in *Y. lipolytica*. The MVA pathway commences with acetyl-CoA, subsequently undergoing multiple reaction steps to generate the precursor FPP. Acetyl-CoA serves as a critical metabolic node, and its availability significantly influences the biosynthesis of terpenes. The efficient conversion of acetyl-CoA to the downstream isoprenoid synthesis often acts as a bottleneck for terpene production in microbial chassis cells [19]. Extensive engineering efforts have been attempted to drive cytosolic acetyl-CoA towards MVA [40]. Given the synergistic effect of MVA pathway throughout the entire continuum, the entire MVA pathway was overexpressed to divert acetyl-CoA toward ABA synthesis. Specifically, tHMG1 was recognized as a critical rate-limiting enzyme in the MVA pathway, with copy number increased to two.

The eight genes in the MVA pathway (Fig. 5A) were distributed among four sets: (1) P_{FBAin}-*tHMG1*-T_{Cyc1t}; (2) P_{EXP}-*IDI*-T_{IDI} and P_{GPD}-*ERG13*-T_{ERG13}; (3) P_{FBAin}-*ERG10*-T_{ERG10}, P_{TEF}-*ERG8*-T_{ERG8}, and P_{FBAin}-*ERG19*-T_{ERG19}; (4) P_{GPD}-*ERG20*-T_{ERG20} and P_{EXP}-*ERG12*-T_{ERG12}.The four sets were iteratively integrated into SM305 strain, resulting in strain SM306-SM309. As shown in Fig. 5B, enhanced ABA production was observed following consecutive genomic integration events. Compared with SM305, the ABA titer in the resulting strains SM306-SM309

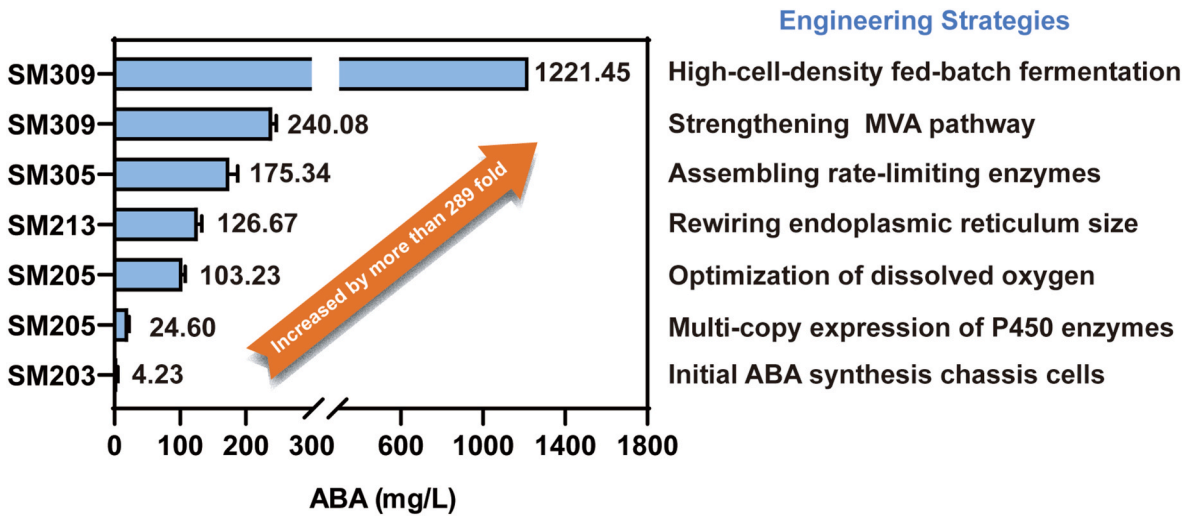


Fig. 7. Overview of the strain engineering process.

exhibited varying degrees of improvement, which exhibited ABA titer that were increased by 6.25 % (186.30 mg/L), 15.89 % (203.21 mg/L), 27.93 % (224.32 mg/L), and 36.90 % (240.08 mg/L), respectively. This study substantiates the significance of synergistic expression of complete MVA pathway, offering valuable insights for the overproduction of other terpenoids.

3.6. *Fed-batch fermentation for abscisic acid production*

To evaluate the potential of SM309 as ABA cell factory, high-density fermentation was carried out. Throughout the 192-h fermentation, glucose concentration, cell growth, and both extracellular and intracellular ABA concentration were monitored every 12 h (Fig. 6). During the fermentation process, glucose was consistently controlled at low levels. At 192 h, the OD_{600} peaked at approximately 324, meeting the requirement for significant product accumulation. The final titer of extracellular and intracellular ABA was 1046.24 and 175.34 mg/L, respectively, corresponding to a yield of 6.10 mg/g glucose and a productivity of 0.025 g/L/h. The results not only underscored the significance of the employed strategy in enhancing ABA synthesis but also demonstrated the remarkable capacity of the engineered *Y. lipolytica* for ABA production. However, there was an observed increase in the intracellular accumulation of ABA in fed-batch fermentation, which may cause a metabolic burden, limiting the development of a highly efficient microbial cell factory.

4. Conclusion

In this study, *Y. lipolytica* was successfully engineered for the accumulation of ABA, scaling up production from milligrams to grams (Supplementary Fig. 1 to Fig. 7). We achieved the highest reported *de novo* ABA production from glucose in a heterologous yeast host by implementing various metabolic engineering strategies, such as constructing a synthetic ABA pathway, addressing rate-limiting steps, and enhancing the MVA pathway (Fig. 7). The strategies described here could serve as a valuable reference for enhancing the production of value-added compounds and improving the functional expression of P450 enzymes in yeasts.

CRediT authorship contribution statement

Mei-Li Sun: Writing – original draft, Visualization, Methodology, Conceptualization. **Ziyun Zou:** Methodology, Data curation. **Lu Lin:** Writing – review & editing, Formal analysis. **Rodrigo Ledesma-Amaro:** Writing – review & editing. **Kaifeng Wang:** Visualization, Investigation, Data curation. **Xiao-Jun Ji:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled, "**Systematic metabolic engineering of *Yarrowia lipolytica* for efficient production of phytohormone abscisic acid**".

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (2217817, 22408166), the Natural Science Found of Jiangsu Province (BK20240539), the Jiangsu Basic Research Center for Synthetic Biology (BK20233003), the China Postdoctoral Science Foundation (2024M751420, GZC20231119), the Jiangsu Agricultural Science and Technology Independent Innovation Fund Project (CX(22)3015), the Jiangsu Synergetic Innovation Center for Advanced Bio-Manufacture (XTC2204), and the Newton Advanced Fellowships (NAF\R1\201187).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2024.10.004.

References

[1] Kato-Noguchi H, Tanaka Y, Murakami T, Yamamura S, Fujihara S. Isolation and identification of an allelopathic substance from peel of Citrus junos. Phytochemistry 2002;61(7):849–53. https://doi.org/10.1016/S0031-9422(02)00382-5.
[2] Finkelstein RR, Gampala SSL, Rock CD. Abscisic acid signaling in seeds and seedlings. Plant Cell 2002;14(suppl_1):S15–45. https://doi.org/10.1105/tpc.010441.
[3] Kermode AR. Role of abscisic acid in seed dormancy. J Plant Growth Regul 2005;24(4):319–44. https://doi.org/10.1007/s00344-005-0110-2.
[4] Mohapatra SS, Poole RJ, Dhindsa RS. Abscisic acid-regulated gene expression in relation to freezing tolerance in alfalfa 1. Plant Physiol 1988;87(2):468–73. https://doi.org/10.1104/pp.87.2.468.
[5] Zeinali YL, Reza H, Fatemeh R, Jalil K. Drought tolerance induced by foliar application of abscisic acid and sulfonamide compounds in tomato. J Stress Physiol Biochem 2014;10(1):326–34.

[6] Gietler M, Fidler J, Labudda M, Nykiel M. Abscisic acid—enemy or savior in the response of cereals to abiotic and biotic stresses? Int J Mol Sci 2020;21(13):4607. https://doi.org/10.3390/ijms21134607.
[7] Booz V, Christiansen CB, Kuhre RE, Saltiel MY, Sociali G, Schaltenberg N, Fischer AW, Heeren J, Zocchi E, Holst JJ. Abscisic acid stimulates the release of insulin and of GLP-1 in the rat perfused pancreas and intestine. Diabetes Metab Res Rev 2019;35(2):e3102. https://doi.org/10.1002/dmrr.3102.
[8] Glennon EKK, Adams LG, Hicks DR, Dehesh K, Luckhart S. Supplementation with abscisic acid reduces malaria disease severity and parasite transmission. Am J Trop Med Hyg 2016;94(6):1266–75. https://doi.org/10.4269/ajtmh.15-0904.
[9] Yamamoto H, Inomata M, Tsuchiya S, Nakamura M, Oritani T. Metabolism of chiral ionylideneacetic acids on the abscisic acid biosynthetic pathway in cercospora. Biosci Biotechnol Biochem 2000;64(12):2644–50. https://doi.org/10.1271/bbb.64.2644.
[10] González-Guzmán M, Apostolova N, Bellés JM, Barrero JM, Piqueras P, Ponce MR, Micol JL, Serrano R, Rodríguez PL. The short-chain alcohol dehydrogenase ABA2 catalyzes the conversion of xanthoxin to abscisic aldehyde. Plant Cell 2002;14(8):1833–46. https://doi.org/10.1105/tpc.002477.
[11] Hirai N, Yoshida R, Todoroki Y, Ohigashi H. Biosynthesis of abscisic acid by the non-mevalonate pathway in plants, and by the mevalonate pathway in fungi. Biosc Biotech Biochem 2000;64(7):1448–58. https://doi.org/10.1271/bbb.64.1448.
[12] Takino J, Kozaki T, Sato Y, Liu C, Ozaki T, Minami A, Oikawa H. Unveiling biosynthesis of the phytohormone abscisic acid in fungi: unprecedented mechanism of core scaffold formation catalyzed by an unusual sesquiterpene synthase. J Am Chem Soc 2018;140(39):12392–5. https://doi.org/10.1021/jacs.8b08925.
[13] Panda S, Zhou K. Engineering microbes to overproduce natural products as agrochemicals. Syn Syst Biotechnol 2023;8(1):79–85. https://doi.org/10.1016/j.synbio.2022.11.005.
[14] Otto M, Teixeira PG, Vizcaino MI, David F, Siewers V. Integration of a multi-step heterologous pathway in Saccharomyces cerevisiae for the production of abscisic acid. Microb Cell Factories 2019;18(1):205. https://doi.org/10.1186/s12934-019-1257-z.
[15] Arnesen JA, Jacobsen IH, Dyekjær JD, Rago D, Kristensen M, Klitgaard AK, Randelovic M, Martinez JL, Borodina I. Production of abscisic acid in the oleaginous yeast Yarrowia lipolytica. FEMS Yeast Res 2022;22(1):foac015. https://doi.org/10.1093/femsyr/foac015.
[16] Liu L, Zhao K, Liu Z. Construction and regulation of the abscisic acid biosynthesis pathway in Yarrowia lipolytica. J Agric Food Chem 2024;72(13):7299–307. https://doi.org/10.1021/acs.jafc.4c00223.
[17] Urlacher VB, Girhard M. Cytochrome P450 monooxygenases in biotechnology and synthetic biology. Trends Biotechnol 2019;37(8):882–97. https://doi.org/10.1016/j.tibtech.2019.01.001.
[18] Hu B, Zhao X, Wang E, Zhou J, Li J, Chen J, Du G. Efficient heterologous expression of cytochrome P450 enzymes in microorganisms for the biosynthesis of natural products. Crit Rev Biotechnol 2022:1–15. https://doi.org/10.1080/07388551.2022.2029344.
[19] Daletos G, Katsimpouras C, Stephanopoulos G. Novel strategies and platforms for industrial isoprenoid engineering. Trends Biotechnol 2020;38(7):811–22. https://doi.org/10.1016/j.tibtech.2020.03.009.
[20] Ji Q, Mai J, Ding Y, Wei Y, Ledesma-Amaro R, Ji X-J. Improving the homologous recombination efficiency of Yarrowia lipolytica by grafting heterologous component from Saccharomyces cerevisiae. Metab Eng Commun 2020:11e00152. https://doi.org/10.1016/j.mec.2020.e00152.
[21] Sun M-L, Han Y, Yu X, Wang K, Lin L, Ledesma-Amaro R, Ji X-J. Constructing a green oleaginous yeast cell factory for sustainable production of the plant-derived diterpenoid sclareol. Green Chem 2024;26(9):5202–10. https://doi.org/10.1039/d3gc04949c.
[22] Su H, Shi P, Shen Z, Meng H, Meng Z, Han X, Chen Y, Fan W, Fa Y, Yang C, Li F, Wang S a. High-level production of nervonic acid in the oleaginous yeast Yarrowia lipolytica by systematic metabolic engineering. Commun Biol 2023;6(1):1125. https://doi.org/10.1038/s42003-023-05502-w.
[23] Ma Y, Li W, Mai J, Wang J, Wei Y, Ledesma-Amaro R, Ji X-J. Engineering Yarrowia lipolytica for sustainable production of the chamomile sesquiterpene (−)-α-bisabolol. Green Chem 2021;23(2):780–7. https://doi.org/10.1039/d0gc03180a.
[24] Wong L, Engel J, Jin E, Holdridge B, Xu P. YaliBricks, a versatile genetic toolkit for streamlined and rapid pathway engineering in Yarrowia lipolytica. Metab Eng Commun 2017:568–77. https://doi.org/10.1016/j.meteno.2017.09.001.
[25] Tong YJ, Zhou JW, Zhang L, Xu P. A golden-gate based cloning toolkit to build violacein pathway libraries in Yarrowia lipolytica. ACS Synth Biol 2021;10(1):115–24. https://doi.org/10.1021/acssynbio.0c00469.
[26] Tai M, Stephanopoulos G. Engineering the push and pull of lipid biosynthesis in oleaginous yeast Yarrowia lipolytica for biofuel production. Metab Eng 2013:151–9. https://doi.org/10.1016/j.ymben.2012.08.007.
[27] Liu X, Cheng J, Zhang G, Ding W, Duan L, Yang J, Kui L, Cheng X, Ruan J, Fan W, Chen J, Long G, Zhao Y, Cai J, Wang W, Ma Y, Dong Y, Yang S, Jiang H. Engineering yeast for the production of breviscapine by genomic analysis and synthetic biology approaches. Nat Commun 2018;9(1):448–57. https://doi.org/10.1038/s41467-018-02883-z.
[28] Shi Y, Dong T, Zeng B, Yao M, Wang Y, Xie Z, Xiao W, Yuan Y. Production of plant sesquiterpene lactone parthenolide in the Yeast cell factory. ACS Synth Biol 2022;11(7):2473–83. https://doi.org/10.1021/acssynbio.2c00132.
[29] Su B, Lai P, Deng M-R, Zhu H. Global rewiring of lipid metabolism to produce carotenoid by deleting the transcription factor genes ino2/ino4 in Saccharomyces cerevisiae. Int J Biol Macromol 2024;264130400. https://doi.org/10.1016/j.ijbiomac.2024.130400.
[30] Kim J-E, Jang I-S, Son S-H, Ko Y-J, Cho B-K, Kim SC, Lee JY. Tailoring the Saccharomyces cerevisiae endoplasmic reticulum for functional assembly of terpene synthesis pathway. Metab Eng 2019:5650–9. https://doi.org/10.1016/j.ymben.2019.08.013.
[31] Silverman AM, Qiao K, Xu P, Stephanopoulos G. Functional overexpression and characterization of lipogenesis-related genes in the oleaginous yeast Yarrowia lipolytica. Appl Microbiol Biotechnol 2016;100(8):3781–98. https://doi.org/10.1007/s00253-016-7376-0.
[32] Arendt P, Miettinen K, Pollier J, De Rycke R, Callewaert N, Goossens A. An endoplasmic reticulum-engineered yeast platform for overproduction of triterpenoids. Metab Eng 2017. https://doi.org/10.1016/j.ymben.2017.02.007. 40165-75.
[33] Xu Y, Wang X, Zhang C, Zhou X, Xu X, Han L, Lv X, Liu Y, Liu S, Li J, Du G, Chen J, Ledesma-Amaro R, Liu L. De novo biosynthesis of rubusoside and rebaudiosides in engineered yeasts. Nat Commun 2022;13(1):3040. https://doi.org/10.1038/s41467-022-30826-2.
[34] Kang W, Ma T, Liu M, Qu J, Liu Z, Zhang H, Shi B, Fu S, Ma J, Lai LTF, He S, Qu J, Wing-Ngor Au S, Ho Kang B, Yu Lau WC, Deng Z, Xia J, Liu T. Modular enzyme assembly for enhanced cascade biocatalysis and metabolic flux. Nat Commun 2019;10(1):4248. https://doi.org/10.1038/s41467-019-12247-w.
[35] Siu K-H, Chen RP, Sun Q, Chen L, Tsai S-L, Chen W. Synthetic scaffolds for pathway enhancement. Curr Opin Biotechnol 2015. https://doi.org/10.1016/j.copbio.2015.08.009. 3698-106.
[36] Zuo R, Zhang Y, Jiang C, Hackett JC, Loria R, Bruner SD, Ding Y. Engineered P450 biocatalysts show improved activity and regio-promiscuity in aromatic nitration. Sci Rep 2017;7(1):842. https://doi.org/10.1038/s41598-017-00897-z.
[37] Zhao F, Bai P, Liu T, Li D, Zhang X, Lu W, Yuan Y. Optimization of a cytochrome P450 oxidation system for enhancing protopanaxadiol production in Saccharomyces cerevisiae. Biotechnol Bioeng 2016;113(8):1787–95. https://doi.org/10.1002/bit.25934.
[38] Zhu H-Z, Jiang S, Wu J-J, Zhou X-R, Liu P-Y, Huang F-H, Wan X. Production of high levels of 3S,3′S-Astaxanthin in Yarrowia lipolytica via iterative metabolic engineering. J Agric Food Chem 2022;70(8):2673–83. https://doi.org/10.1021/acs.jafc.1c08072.
[39] Jia F, Narasimhan B, Mallapragada S. Materials-based strategies for multi-enzyme immobilization and co-localization: a review. Biotechnol Bioeng 2014;111(2):209–22. https://doi.org/10.1002/bit.25136.
[40] Liu Q, Zhang G, Su L, Liu P, Jia S, Wang Q, Dai Z. Reprogramming the metabolism of oleaginous yeast for sustainably biosynthesizing the anticarcinogen precursor germacrene A. Green Chem 2023;25(20):7988–97. https://doi.org/10.1039/d3gc01661g.