Metabolic engineering of *Saccharomyces cerevisiae* for enhanced production of caffeic acid

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Received: 2 February 2021 / Revised: 9 June 2021 / Accepted: 3 July 2021 / Published online: 20 July 2021

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Abstract

As a natural phenolic acid product of plant source, caffeic acid displays diverse biological activities and acts as an important precursor for the synthesis of other valuable compounds. Limitations in chemical synthesis or plant extraction of caffeic acid trigger interest in its microbial biosynthesis. Recently, *Saccharomyces cerevisiae* has been reported for the biosynthesis of caffeic acid via episomal plasmid-mediated expression of pathway genes. However, the production was far from satisfactory and even relied on the addition of precursor. In this study, we first established a controllable and stable caffeic acid pathway by employing a modified GAL regulatory system to control the genome-integrated pathway genes in *S. cerevisiae* and realized biosynthesis of 222.7 mg/L caffeic acid. Combinatorial engineering strategies including eliminating the tyrosine-induced feedback inhibition, deleting genes involved in competing pathways, and overexpressing rate-limiting enzymes led to about 2.6-fold improvement in the caffeic acid production, reaching up to 569.0 mg/L in shake-flask cultures. To our knowledge, this is the highest ever reported titer of caffeic acid synthesized by engineered yeast. This work showed the prospect for microbial biosynthesis of caffeic acid and laid the foundation for constructing biosynthetic pathways of its derived metabolites.

Key points

- Genomic integration of ORgTAL, OHpaB, and HpaC for caffeic acid production in yeast.
- Feedback inhibition elimination and Aro10 deletion improved caffeic acid production.
- The highest ever reported titer (569.0 mg/L) of caffeic acid synthesized by yeast.

Keywords Caffeic acid · *Saccharomyces cerevisiae* · GAL regulatory system · Tyrosine-induced feedback inhibition

Introduction

Caffeic acid, also known as 3,4-dihydroxycinnamic acid, has attracted increasing attention due to its antioxidant (Chen and Ho 1997), antiviral (Ikeda et al. 2011), anticancer (Espindola et al. 2019; Rajendra Prasad et al. 2011), and anti-inflammatory biological properties (Chao et al. 2009). Moreover, caffeic acid is an important precursor of plant-originated aromatic chemicals such as rosmarinic acid, chlorogenic acid, and caffeic acid phenethyl ester (Bloch and Schmidt-Dannert 2014; Murthy et al. 2014; Wang et al. 2017). Therefore, it shows great potential in the nutritional, pharmaceutical, and cosmetics industries (Magnani et al. 2014). Considering the environmental and economic benefits, biosynthesis of caffeic acid via engineering model microbes such as *Escherichia coli* and *Saccharomyces cerevisiae* provides a promising alternative to chemical synthesis or plants extraction (Cao et al. 2020).

The biosynthesis of caffeic acid starts from L-phenylalanine or L-tyrosine through the endogenous shikimate pathway (Fig. 1) (Liu et al. 2019a). In plants, the deamination of L-phenylalanine is catalyzed by phenylalanine...
ammonia lyase (PAL) to produce cinnamic acid. The sequential two-step hydroxylation at the 4- and 3-positions of the benzyl ring of cinnamic acid is executed by two cytochrome P450 monooxygenases, cinnamate-4-hydroxylase (C4H), and p-coumarate 3-hydroxylase (C3H) (Kim et al. 2011), forming caffeic acid through p-coumaric acid. In recent years, reports have emerged regarding metabolic engineering for heterogeneous caffeic acid production in *E. coli*. However, the plant-originated P450 enzymes are difficult to express in microbial systems (Lin and Yan 2012). Alternatively, tyrosine containing a 4-hydroxyl group could be directly converted to p-coumaric acid by microbial tyrosine ammonia lyase (TAL) (Hernández-Chávez et al. 2019). For further hydroxylation of p-coumaric acid, the *sam5*-encoded *Coum3H* from the actinomycete *Saccharothrix espanaensis* (Berner et al. 2006; Choi et al. 2011) or the cytochrome P450 CYP199A2 from the bacteria *Rhodopseudomonas palustris* (Furuya et al. 2012; Rodrigues et al. 2015) could be used, enabling caffeic acid formation in *E. coli*. Huang et al. (2013) and Jones et al. (2016) revealed a high affinity of *E. coli* hydroxylase complex 4HPA3H (also named HpaBC) towards p-coumaric acid and more than 3 g/L of caffeic acid production from a feed of 4 g/L p-coumaric acid in HpaBC-overexpressed *E. coli*, respectively. By introducing *RgTAL* from *Rhodotorula glutinis* into *E. coli* together with overexpressing the endogenous 4-hydroxyphenylacetate 3-hydroxylase (4HPA3H) and increasing the intracellular supply of tyrosine by overexpression of PEP synthase (encoded by ppsA), transketolase (encoded by tktA), feedback-inhibition-resistant 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (encoded by *aroG*<sup>tiea</sup>),

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Fig. 1 The biosynthetic pathway of caffeic acid from glucose in *S. cerevisiae*. EMP pathway, Embden-Meyerhof-Parnas pathway; PPP pathway, pentose phosphate pathway; PEP, phosphoenolpyruvate; E4P, 4-erythritol phosphate; DAHP, 3-deoxy-D-arabino-heptulosonate-7-phosphate; L-Tyr, tyrosine; 4-HPP, p-hydroxyphenylpyruvate; 4HPAA, p-hydroxyphenylacetaldehyde; L-Trp, tryptophan; PPY, phenylpyruvate; PAC, phenylacetaldehyde; L-Phe, Phenylalanine; L-Tyr, tyrosine; 4-HPP, p-hydroxyphenylpyruvate; 4HPAA, 4-hydroxy-phenylacetaldehyde. Dotted lines indicate multi-enzyme reactions.
and chorismate mutase-prephenate dehydrogenase (encoded by tyrA<sup>PP</sup>), the caffeic acid production reached 766.7 mg/L in shake flasks without p-coumaric acid addition (Huang et al. 2013). However, both the cell growth and caffeic acid production relied on phenylalanine supplementation, which is not cost-effective in industrial production. Overexpression of RgTAL and EcHpaBC in tyrosine-overproducing <i>E. coli</i> strain ppoA14(DE3) together with optimization of culture conditions resulted in the highest titer (1.03 g/L) of caffeic acid from simple carbon to date (Jones et al. 2017).

<i>S. cerevisiae</i> as GRAS (generally regarded as safe) organism with well-characterized genetic background, superior stress tolerance, and excellent fermentation properties has recently become another attractive microbial host for caffeic acid production. The <i>E. coli</i> 4HPA3H complex encoded by HpaB and HpaC was found to effectively catalyze p-coumaric acid to caffeic acid in <i>S. cerevisiae</i> (Lin and Yan 2012). When the <i>E. coli</i> enzymes were replaced with the combination of HpaB from <i>Pseudomonas aeruginosa</i> and HpaC from <i>Salmonella enterica</i>, the caffeic acid yield was significantly improved by 45.9-fold, leading to the highest caffeic acid production (289.4 mg/L) in yeast (Liu et al. 2019a). However, this process relied on the feeding of exogenous L-tyrosine as the precursor. Li et al. reported that simultaneous expression of RcTAL from <i>Rhodobacter capsulatus</i> and the P450-dependent monooxygenase C3H together with its associated cytochrome P450 reductase CPR1 from <i>Arabidopsis thaliana</i> could enable de novo biosynthesis of caffeic acid from glucose in <i>S. cerevisiae</i> without the need of precursor supplementation. However, low caffeic acid production (11.4 mg/L) was obtained, ascribed to the low activity of C3H (Li et al. 2020). In both studies, episomal vectors were used for the expression of caffeic acid pathway genes in <i>S. cerevisiae</i>. Considering that the yeast transformants harboring several plasmids are genetically unstable, integrating the caffeic acid pathway into the yeast genome may create a more stable cell factory.

As found with other tyrosine-derived products (Gottardi et al. 2017; Lyu et al. 2017; Rodriguez et al. 2015), the shortage of precursor supply may be another limiting factor of caffeic acid biosynthesis in <i>S. cerevisiae</i>. The critical step of the shikimate pathway is the condensation of two starter units named phosphoenolpyruvate (PEP) and 4-erythritol phosphate (E4P) by isoenzymes Aro3 and Aro4 to produce 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) (Gao et al. 2017; Gottardi et al. 2017). In addition, chorismate, the last common intermediate for three aromatic amino acids, is transformed by the chorismate mutase Aro7 to generate prephenate, which is further divided into two branches, one towards L-phenylalanine and the other towards L-tyrosine (Fig. 1). In this pathway, the activities of Aro4 and Aro7 are feedback inhibited by the end product tyrosine. Feedback-insensitive variants Aro4<sup>K229L</sup> and Aro7<sup>G141S</sup> have been created by rational design (Hartmann et al. 2003; Luttik et al. 2008; Schnappauf et al. 1998). Either individual overexpression of Aro4<sup>K229L</sup> or simultaneous expression of Aro4<sup>K229L</sup> and Aro7<sup>G141S</sup> could effectively increase intracellular shikimate, phenylalanine, and tyrosine concentrations (Gao et al. 2017; Luttik et al. 2008). The prephenate dehydrogenase (Tyr1) which catalyzes prephenate to α-keto acid 4-hydroxyphenylpyruvate (4HPP), the direct precursor of L-tyrosine, is less sensitive to tyrosine inhibition (Lopez-Nieves et al. 2019) but transcriptionally inhibited by phenylalanine (Gold et al. 2015; Mannhaupt et al. 1989). Replacement of the native Tyr1 promoter with a constitutive one or expression of the feedback-insensitive cyclohexadienyl dehydrogenase TyrC from <i>Zymomonas mobilis</i> could both improve the production of tyrosine and its derivatives (Gold et al. 2015; Mao et al. 2017). Meanwhile, decarboxylation of 4HPP catalyzed by phenylpyruvate decarboxylases (encoded by Aro10, Pdc1, Pdc5, and Pdc6) would decrease the flux towards tyrosine, among which Pdc5 and Aro10 showed stronger decarboxylation activity than the others (Romagnoli et al. 2012). Deletion of Aro10 and Pdc5 increased the intracellular tyrosine production by 5.7 folds (Lyu et al. 2017).

Taken together, alleviation of feedback inhibition and removal of competitive branches could improve the precursor supply and thus contribute to further enhancement of caffeic acid synthesis. In this study, we first constructed a controllable and stable caffeic acid biosynthetic pathway in <i>S. cerevisiae</i> by employing a modified GAL system to control the genome-integrated genes. The precursor supply was then strengthened by eliminating the feedback inhibition of aromatic amino acid and downregulating the competitive pathways, and the rate-limiting enzyme TyrC was overexpressed to enhance the flux towards caffeic acid production. The combinatorial engineering strategy enabled efficient biosynthesis of caffeic acid in yeast, with caffeic acid titer reaching up to 569.0 mg/L in shake flasks.

### Materials and methods

#### Strain, media, and reagents

<i>E. coli</i> DH5α (Novagen, USA) was used for propagation of recombinant plasmids. Luria-Bertani (LB) broth medium (0.5% Bacto yeast extract, 1% Bacto-tryptone [Difco Laboratories], 1% NaCl, pH 7.0) containing 50 μg/mL of kanamycin or 100 μg/mL of ampicillin was used for culturing <i>E. coli</i> carrying transformed vectors. <i>S. cerevisiae</i> strain BY4741 was used as the host for constructing the caffeic acid biosynthetic pathway. YPD medium (1% yeast extract, 2% peptone, and 2% glucose) was used for routine cultivation of <i>S. cerevisiae</i> strains. Geneticin (G418, 200 μg/mL) was supplemented in a YPD agar plate for the selection of engineered
yeast strains. SC agar plates (synthetic complete drop-out medium supplemented with 20 g/L D-glucose, 5 g/L ammonium sulfate, 1.7 g/L yeast nitrogen base, 100 mg/L uracil, 100 mg/L L-histidine-HCL, 100 mg/L L-methionine, 150 mg/L L-leucine, 1.5% agar) containing 1 mg/mL of 5-fluoroorotic acid were used for selection of recombinants with KanMX-URA3-PRB322ori marker excision. In addition, SD-URA medium consists of 20 g/L D-glucose, 5 g/L ammonium sulfate, 1.7 g/L yeast nitrogen base, 100 mg/L L-histidine-HCL, 100 mg/L L-methionine, and 150 mg/L L-leucine.

Deletion of Aro3, Aro10, and Pdc5 genes

Aro3-UP homologous arm of Aro3 was amplified with the primers Aro3-upF and Aro3-upR. The downstream homologous arm of Aro3 was amplified with the primers Aro3-DnF and Aro3-DnR, generating Aro3-Down. The Aro3 homologous arms were obtained by overlap extension PCR using Aro3-upF and Aro3-DnR as the primers and Aro3-UP and Aro3-Down as the templates. The resulting segment was fused with plasmid backbone amplified from pUMRI-11 (KM216413) (Lv et al. 2016) with the primers p21-F1 and p21-R1 by in vitro homologous recombination, generating pUMRI-ΔAro3. Similarly, pUMRI-ΔAro10 and pUMRI-ΔPdc5 plasmids containing the homologous arms of Aro10 and Pdc5 respectively were constructed. The recombinant plasmids were digested with SfiI and integrated into the corresponding loci to delete Aro3, Aro10, or Pdc5. The primers are listed in Table S1.

Genes amplification and recombinant plasmids construction

For construction of the caffeic acid synthetic pathway, ORgTAL (MW623639) from R. glutinis, OHpaB (MW623641) from P. aeruginosa, and OTyrC (MW623640) from Z. mobilis were codon-optimized according to the preferred codon usage of S. cerevisiae using JCAT tools (http://www.jcat.de/) and synthesized by Generalay Biotech (Shanghai, China). HpaC (MW263642) was amplified from S. enterica C50336 using HpaC-F (EcoR I) and HpaC-R (Bgl II) primers. The detailed sequences of ORgTAL, OHpaB, HpaC, and OTyrC are shown in the supporting information. Aro4 and Aro7 were amplified from the genome of S. cerevisiae. Single-site mutants of Aro4 and Aro7 were created by overlap extension PCR according to the previous report (Urban et al. 1997). The series of pUMRI plasmids were used to integrate the pathway genes into different genomic loci of S. cerevisiae. The detailed information on plasmids used in this study are listed in Table 1.

Construction of caffeic acid-producing yeast strains

The starting strain YXWP-113 was constructed in previous work by knocking out the GAL80 gene of BY4741 via homologous recombination and using the LEU2 marker for auxotroph selection (Zhou et al. 2017). The recombinant pUMRI plasmids carrying pathway genes linearized with SfiI were sequentially transformed into YXWP-113 using the LiAc/SS carrier DNA/PEG method (Gietz and Schiestl 2007), and the resulting strains were selected on YPD agar plate containing G418 (200 μg/mL). The genes were integrated into different genomic loci, such as GAL1-7 (ChrII 274632-279536), DPPI (ChrIV 1031419-1030550), Aro3 (Chr IV 521939-522785), Aro10 (Chr IV 1233993-1235961), and Pdc5 (Chr XII 410723-412414). The detailed information of the yeast strains are shown in Table 2.

Cultivation in shaking flasks for caffeic acid production

Single colonies were picked from the YPD agar plate, inoculated into 5 mL YPD culture tubes, and incubated overnight at 30°C in a rotary shaker (220 rpm). The seed cultures were then transferred into a 50 mL fresh YPD medium without tyrosine addition to an initial OD600 of 0.05 and grown under the same conditions for 72 h. For measurement of dry cell weight, 2 mL cultures were harvested by centrifugation at 12,000 rpm for 2 min, and yeast cells were washed twice and then dried at 95°C to a constant weight.

Analysis of the metabolites

For quantification of p-coumaric acid, caffeic acid, and tyrosol, 500 μL of the culture was mixed thoroughly with an equal volume of methanol. The supernatant mixture was collected by centrifugation at 12,000 rpm for 5 min and then diluted with 50% methanol. After filtered through 0.22 μm filters, the samples were analyzed on an HPLC (Agilent 1200) system equipped with Pntulips QS-C18 PLUS column (4.6 × 250 mm, 5 μm, puningtech). The gradient program was performed with solvent A (water containing 0.1% (v/v) formic acid) and solvent B (acetonitrile) as the mobile phase. The program started with 90% of solvent A and 10% of solvent B, the concentration of solvent B subsequently increased to 30% within 15 min and continued up to 50% at 30 min. The flow rate was 1 mL/min with a constant column temperature at 35°C. Tyrosol, caffeic acid, and p-coumaric acid were detected at 9.7 min (276 nm), 11.5 min (320 nm), and 14.8 min (308 nm), respectively.

The production of 2-phenylethanol was quantified by gas chromatography (GC). The culture (100 μL) was mixed thoroughly with 900 μL methanol and centrifuged at 12,000 rpm for 5 min. One μL of diluted supernatants was injected into gas chromatography (Fuli, Wenling, China) equipped with an
Results

Construction of a glucose-regulated caffeic acid biosynthetic pathway in S. cerevisiae

Biosynthesis of caffeic acid could be derived from tyrosine, while S. cerevisiae does not possess the pathway downstream of tyrosine. For heterologous biosynthesis of caffeic acid, RgTAL from R. gruminis, HpaB from P. aeruginosa, and

Table 1  Plasmids constructed in this study

| Plasmids | Genotype/description | Reference |
|----------|----------------------|-----------|
| pUMRI-11 | loxp-KanMX::URA3-pbr322ori-loxp, TADH1-MCS1-Pgall10-Pgall2-TCYC1, DPP1 | KM216413 |
| pUMRI-13 | loxp-KanMX::URA3-pbr322ori-loxp, TADH1-MCS1-Pgall10-Pgall2-TCYC1, GAL1-7 | KM216415 |
| PUMRI-13-OhpAB-HpaC | loxp-KanMX::URA3-pbr322ori-loxp, TADH1-HpaC-Pgall10-Pgall2-OhpAB-TCYC1, GAL1-7 | This study |
| PUMRI-11-OrTAL | loxp-KanMX::URA3-pbr322ori-loxp, TADH1-MCS1-Pgall10-Pgall2-OrTAL-TCYC1, DPP1 | This study |
| pUMRI-Δmm3 | loxp-KanMX::URA3-pbr322ori-loxp, TADH1-MCS1-Pgall10-Pgall2-TCYC1, ArO3 | This study |
| pUMRI-Δmm10 | loxp-KanMX::URA3-pbr322ori-loxp, TADH1-MCS1-Pgall10-Pgall2-TCYC1, ArO10 | This study |
| pUMRI-Δmm3-AroK229L | loxp-KanMX::URA3-pbr322ori-loxp, TADH1-MCS1-Pgall10-Pgall2-AroK229L-TCYC1, ArO3 | This study |
| pUMRI-Δmm10-AroK229L-AroK141S | loxp-KanMX::URA3-pbr322ori-loxp, TADH1-MCS1-Pgall10-Pgall2-AroK229L-AroK141S-TCYC1, ArO3 | This study |
| pUMRI-Δmm10-OtYcC | loxp-KanMX::URA3-pbr322ori-loxp, TADH1-MCS1-Pgall10-Pgall2-AroK229L-AroK141S-TCYC1, ArO3 | This study |
| pUMRI-Δmm10-ΔPd5 | loxp-KanMX::URA3-pbr322ori-loxp, TADH1-MCS1-Pgall10-Pgall2-TCYC1, Pd5 | This study |

* MCS represents multiple cloning sites used for gene insertion

Table 2  Strains used in this study

| Strain | Genotype/description | Reference |
|--------|----------------------|-----------|
| BY4741 | MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 | (Brachmann et al. 1998) |
| YXWP-113 | BY4741, Δgal80::LEU2 | (Xie et al. 2015) |
| Yp·CA-2 | YXWP-113, Δdpp1:::TADH1-MCS1-Pgall10-Pgall2-OrTAL-TCYC1-KanMX-URA3-PBR322ori | This study |
| YCA113-1B | YXWP-113, Δgal1-7::TADH1-HpaC-Pgall10-Pgall2-OhpAB-TCYC1 | This study |
| YCA113-1B | YXWP-113, Δgal1-7::TADH1-HpaC-Pgall10-Pgall2-OhpAB-TCYC1, Δdpp1:::TADH1-MCS1-Pgall10-Pgall2-OrTAL-TCYC1-KanMX-URA3-PBR322ori | This study |
| YCA113-2B | YXWP-113, Δgal1-7::TADH1-HpaC-Pgall10-Pgall2-OhpAB-TCYC1, Δdpp1:::TADH1-MCS1-Pgall10-Pgall2-OrTAL-TCYC1-KanMX-URA3-PBR322ori | This study |
| YCA113-3B | YXWP-113, Δgal1-7::TADH1-HpaC-Pgall10-Pgall2-OhpAB-TCYC1, Δdpp1:::TADH1-MCS1-Pgall10-Pgall2-OrTAL-TCYC1-KanMX-URA3-PBR322ori | This study |
| YCA113-4B | YXWP-113, Δgal1-7::TADH1-HpaC-Pgall10-Pgall2-OhpAB-TCYC1, Δdpp1:::TADH1-MCS1-Pgall10-Pgall2-OrTAL-TCYC1-KanMX-URA3-PBR322ori | This study |
| YCA113-5B | YXWP-113, Δgal1-7::TADH1-HpaC-Pgall10-Pgall2-OhpAB-TCYC1, Δdpp1:::TADH1-MCS1-Pgall10-Pgall2-OrTAL-TCYC1-KanMX-URA3-PBR322ori | This study |
| YCA113-6B | YXWP-113, Δgal1-7::TADH1-HpaC-Pgall10-Pgall2-OhpAB-TCYC1, Δdpp1:::TADH1-MCS1-Pgall10-Pgall2-OrTAL-TCYC1-KanMX-URA3-PBR322ori | This study |
| YCA113-7B | YXWP-113, Δgal1-7::TADH1-HpaC-Pgall10-Pgall2-OhpAB-TCYC1, Δdpp1:::TADH1-MCS1-Pgall10-Pgall2-OrTAL-TCYC1-KanMX-URA3-PBR322ori | This study |
| YCA113-8B | YXWP-113, Δgal1-7::TADH1-HpaC-Pgall10-Pgall2-OhpAB-TCYC1, Δdpp1:::TADH1-MCS1-Pgall10-Pgall2-OrTAL-TCYC1-KanMX-URA3-PBR322ori | This study |
| YCA113-9B | YXWP-113, Δgal1-7::TADH1-HpaC-Pgall10-Pgall2-OhpAB-TCYC1, Δdpp1:::TADH1-MCS1-Pgall10-Pgall2-OrTAL-TCYC1-KanMX-URA3-PBR322ori | This study |
| YCA113-10B | YXWP-113, Δgal1-7::TADH1-HpaC-Pgall10-Pgall2-OhpAB-TCYC1, Δdpp1:::TADH1-MCS1-Pgall10-Pgall2-OrTAL-TCYC1-KanMX-URA3-PBR322ori | This study |

* MCS represents multiple cloning sites used for gene insertion
HpaC from *S. enterica* reported with excellent performance (Liu et al. 2019a; Santos et al. 2011) were chosen as the target genes for pathway construction. To avoid the expensive galactose addition, the modified *GAL* system constructed by knocking out *GAL80* (encoding a repressor of Gal4, which confers repression in the absence of galactose) was employed herein, leading to dynamic expression of the target genes in response to glucose concentration (Fig. 2a). Firstly, the codon-optimized ORgTAL under *GAL1* promoter was integrated into the *DPP1* site of the *GAL80*-knockout strain YXWP-113 (Xie et al. 2015), and the resulting strain Yp-CA-2 produced 116.7 mg/L of *p*-coumaric acid in YPD medium without precursor addition (Fig. 2b). To further convert *p*-coumaric acid to caffeic acid, the codon-optimized *OHpaB* together with *HpaC* under *GAL1/GAL10* promoters were integrated into the *GAL1*-7 genomic loci of Yp-CA-2. The metabolites of the resulting strain YCA113-2B were analyzed by high-performance liquid chromatography (HPLC). Obviously decreased accumulation of *p*-coumaric acid was observed together with a newly emerging peak which had the same retention time as the caffeic acid standard when comparing the spectra to those of Yp-CA-2 (Fig. 2c). Almost 90.8% of *p*-coumaric acid was successfully transformed to caffeic acid, with only 10.7 mg/L of *p*-coumaric acid remained (Fig. 2b). Time courses of YCA113-2B showed that extracellular glucose could not be detected after 12 h of cultivation, while the biomass and caffeic acid production has continued to increase after glucose depletion. After uptake into the cells, glucose was transformed into glycolytic intermediates and then catabolized to CO2 and ethanol. We assumed yeast could continue to use the glycolytic intermediates and ethanol for biomass production and caffeic acid synthesis. Therefore, the accumulation of ethanol in YCA113-2B during the process of fermentation was analyzed (Fig. 2d). The results were in accordance with the previous assumption that the ethanol continued to accumulate until glucose depletion and the 15 g/L of ethanol has been produced at 12 h. Subsequently, the concentration of ethanol was decreased that it was then used as the carbon source for biomass production and caffeic acid synthesis (Fig. 2d). Finally, 222.7 mg/L of caffeic acid was accumulated after 72 h of shake-flask culture in the medium without any precursor added.

Elimination of feedback inhibition of DAHP synthases and chorismate mutase

The biosynthesis of tyrosine, the direct precursor of caffeic acid, is strictly regulated in *S. cerevisiae*. Therefore, relieving feedback inhibition of tyrosine may lead to improved caffeic acid production. After deletion of *Aro3* gene inhibited by phenylalanine, little effect on *p*-coumaric acid, caffeic acid accumulation, and cell growth was observed (Fig. 3). Thus, *Aro3* can be used as a target locus for gene integration. Subsequently, tyrosine feedback inhibition insensitive mutant *Aro4*K229L under *GAL1* promoter was integrated into *Aro3* site by using the linearized PUMR-Δ*Aro3-Aro4*K229L vector. The resulting strain YCA113-4B produced 330.4 mg/L of caffeic acid with 21.3 mg/L of *p*-coumaric acid remained unconverted, which was about 67.8% and 83.6% higher than those before *Aro4*K229L overexpression (Fig. 3). However, the biomass of YCA113-4B decreased by 10.2% compared with YCA113-3B.

In addition, chorismate mutase *Aro7* is another key enzyme feedback inhibited by tyrosine. Therefore, the tyrosine-insensitive variant *Aro7*G141S was overexpressed in YCA113-4B strain, which slightly increased the caffeic acid titer (341.8 mg/L) and *p*-coumaric acid level (24.7 mg/L) in the resulting strain YCA113-5B. The production improvement after the alleviation of feedback inhibition regulation was not as significant as anticipated, implying the presence of downstream bottlenecks.

Deletion of competing pathway genes and overexpression of rate-limiting caffeic acid synthetic enzymes

To further improve the production of caffeic acid, the rate-limiting steps in the synthetic pathway were eliminated by overexpression of the corresponding enzymes, and the carbon flux was redirected to the target pathway by deletion of the competitive pathways. Phenylpyruvate decarboxylase (Aro10) is a key enzyme responsible for the degradation of aromatic amino acids to fusel alcohols such as 2-phenylethanol and tyrosol via the Ehrlich pathway. Thus, *Aro10* gene was deleted in the YCA113-5B strain to reduce the competitive consumption of the precursors. The resulting strain YCA113-6B produced about 435.2 mg/L of caffeic acid and 30.6 mg/L of *p*-coumaric acid, which was improved by 27.3% and 23.9% compared with YCA113-5B. The 2-phenylethanol production in YCA113-6B with *Aro10* deletion (21.3 mg/L) was notably lower than that of YCA113-5B without *Aro10* deletion (94.3 mg/L) (Fig. S1a). In contrast, no tyrosol was accumulated in caffeic acid-producing strains either in the presence or absence of *Pdc5* and *Aro10* (Fig. S1b), which implied the higher competitiveness of the caffeic acid biosynthetic pathway against the tyrosol-forming branch.

In addition, the transcription of the native *Tyr1* gene is tightly regulated by phenylalanine concentration (Mao et al. 2017), which limited the conversion of prephenate to 4-HPP. Therefore, feedback-insensitive *TyrC* from *Z. mobilis* was integrated into the *Aro10* genomic loci of YCA113-6B to deregulate this reaction. The resulting strain YCA113-8B produced 569.0 mg/L of caffeic acid, which was 30.7% and 156% higher than those of YCA113-6B strain and YCA113-2B, respectively (Fig. 3). Meanwhile, 35.3 mg/L of *p*-coumaric acid remained unconverted. 2-Phenylethanol production of YCA113-8B was also slightly decreased as
compared with YCA113-6B (Fig. S1a). When we further deleted the aromatic amino acid-consuming pyruvate decarboxylase (encoded by Pdc5) in strain YCA113-8, no significant production improvement was observed in the resulting strain YCA113-10, and there was little effect on 2-phenylethanol concentration. We speculated that Pdc6 and Pdc1 also have decarboxylase activity on aromatic amino acid degradation (Dickinson et al. 2003; Koopman et al. 2012; Romagnoli et al. 2012).

**Discussion**

In this work, we aim to construct an efficient caffeic acid-producing cell factory for the biosynthesis of this valuable product using *S. cerevisiae* as chassis, which could as well serve as a basis for future biosynthesis of its derivatives such as rosmarinic acid. In the previous work of Li et al. (2020) and Liu et al. (2019a), episomal plasmids carrying the caffeic acid pathway genes were applied for construction of caffeic acid-producing *S. cerevisiae*. However, this may cause problems such as plasmid instability, excessive metabolic burden, and requirement of selective pressure. As compared to the previous reports on engineering yeast for caffeic acid production, the novelty of this work lies in the genomic integration of pathway genes under the regulation of a modified GAL system for stable and controllable expression and in the independence from external precursor addition by strengthening endogenous precursor supply. To pull more carbon resources to caffeic acid biosynthesis, we introduced the HpaB from...
Pseudomonas aeruginosa and HpaC from Salmonella enteritidis which were reported with high efficiency (Liu et al. 2019a). The combination of RgTAL from R. glutinis, HpaB from P. aeruginosa, and HpaC from S. enterica was first reported for assembly of caffeic acid pathway in microbial cells, which showed superior performance over the previously reported gene combinations.

Previous reports showed that the biomass and resveratrol, p-coumaric acid, and caffeic acid production in the YPD medium were much higher than those in the simple medium (Li et al. 2020; Liu et al. 2017). However, some studies had to use a synthetic complete (SC) medium lacking relevant amino acids to facilitate the selection of strains containing plasmids (Gupta and Mukherjee 2002; Li et al. 2020; Silva 1993). In our work, the caffeic acid pathway genes were integrated into the yeast genome, which was stable in the YPD medium without selective pressure. To provide a direct comparison of cultures in complex and simple media, we have also cultured the engineered strains in SD-URA medium. The variation trends of caffeic acid production among different strains in the SD-URA medium were similar to those in the YPD medium (Fig. S2), while the biomass and caffeic acid production in SD-URA medium were much lower than those in YPD medium. Therefore, the complex YPD medium containing all types of amino acids was adopted in our study.
Caffeic acid production of the strain YCA113-2B (expressing RgTAL, OHpaB, and HpaC) was much higher than the p-coumaric acid accumulation in the strain Yp-CA-2 (expressing only ORgTAL), which was similar to the observation of Liu et al. (2019a), where 117.5 mg/L of p-coumaric acid was produced in the strain expressing RgTAL from R. toruloides with supplementation of 500 mg/L of tyrosine and 289.4 mg/L of caffeic acid was produced in the strain expressing HpaB from Pseudomonas aeruginosa and HpaC from Salmonella enterica together with RgTAL. The extension of the biosynthetic pathway by introducing p-coumaric acid converting enzymes could also pull the metabolic flux towards the final product caffeic acid. Moreover, the higher yield of caffeic acid in the final strain than the p-coumaric acid yield in the intermediate-accumulating strain might be ascribed to the presence of another synthetic route to convert L-tyrosine to caffeic acid. In a previous study, Lin and Yan (2012) demonstrated that caffeic acid can not only be formed from L-tyrosine via p-coumaric acid by sequential catalysis of TAL and 4HPA3H, but also from L-tyrosine via L-dopa by 4HPA3H and TAL in an opposite catalysis order. The darkened color was observed from YCA113-1B (integrating OHpaB and HpaC in XYWP113) and YCA113-2B in liquid medium (Fig. S3). This is consistent with a previous report that the unstable intermediate L-dopa formed from tyrosine by OHpaB and HpaC is readily oxidized to melanin, causing darker coloration of the fermentation broth (Chen et al. 2017; Fordjour et al. 2019; Huang et al. 2013). In addition, we can deduce from the lighter color of the culture broth of YCA113-2B compared with YCA113-1B that the oxidation of L-dopa was reduced in YCA113-2B. This implied the existed competitiveness of the caffeic acid biosynthetic pathway against the L-dopa oxidized branch. To further demonstrate the possibility of conversion of L-dopa to caffeic acid, Yp-CA-2 (expressing only ORgTAL in XYWP-113) and XYWP-113 were cultured with 200 mg/L L-dopa addition. The result showed 4.4 mg/L of caffeic acid was accumulated in Yp-CA-2 cultures, while no caffeic acid was detected in the cultures of XYWP-113 which indicated that ORgTAL was able to convert L-dopa. However, a large portion of the added L-dopa was oxidized, leading to the darkening color of the fermentation broth. We could not provide firm evidence for an explanation of the preference for the two synthetic routes at the moment. Considering side reactions of the L-dopa synthetic route, channeling carbon flux through the p-coumaric acid synthetic route is more economical for caffeic acid production.

The influence of Pdc5/Aro10 double deletion on the biomass differed among strains with different genetic backgrounds. Our result was consistent with the report of Li et al. (2020) where the cell growth was also not affected in Pdc5/Aro10 double-knockout caffeic acid-producing S. cerevisiae. In another report, Lyu et al. (2017) showed a slight improvement of biomass after deletion of Pdc5 and Aro10 in naringenin-producing yeast, while Liu et al. (2019b) reported that the introduction of Pdc5 and Aro10 deletions indeed led to negative impact on cell growth. However, the degree of influence was different in different strains. The strain expressing the phosphoketolase-based pathway and optimized by using a GAL control system showed little change in cell growth after Pdc5 and Aro10 deletions. The little influence of Pdc5 deletion on caffeic acid production in this study might be caused by the insufficient downstream pathway in charge of converting the precursors to caffeic acid. Therefore, strengthening the downstream pathway may be a viable approach to further improvement of caffeic acid production. In this study, the highest ever reported caffeic acid production (569.0 mg/L) in engineered yeast S. cerevisiae was realized via the relief of tyrosine feedback inhibition, reduction of carbon flux diversion into competing pathways, and overexpression of rate-limiting pathway enzymes. This work highlights the benefit of strengthening the precursor flux and demonstrates the importance of eliminating downstream rate-limiting steps in caffeic acid biosynthesis. The yeast strains obtained in this work would lay a foundation for microbial hyperproduction of caffeic acid and its derivatives.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00253-021-11445-1.

Author contribution PPZ conceived and designed the experiments. PPZ and CLY performed the experiments. BS, YD, and NNX assisted in experiments. PPZ wrote and edited the manuscript. LDY contributed materials and revised the manuscript. All authors read and approved the final manuscript.

Funding This work was financially supported by the National Natural Science Foundation of China (Grant No. 32001032), Natural Science Foundation of Jiangsu Province, China (Grant No. BK20200946), China Postdoctoral Science Foundation (Grant No. 2020M671614), Natural Science Research of Jiangsu Higher Education Institutions of China (20KJD416003), the Innovative and Entrepreneurial Talent of Jiangsu Province, and “Lvyang Jinfeng” Talent Attracting Plan of Yangzhou.

Data availability All experimental data and strains constructed in this study will be made available from the corresponding author upon reasonable request from readers.

Declarations

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare no competing interests.
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