



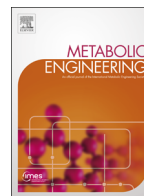
## **Establishment of a yeast platform strain for production of p-coumaric acid through metabolic engineering of aromatic amino acid biosynthesis**

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# Establishment of a yeast platform strain for production of *p*-coumaric acid through metabolic engineering of aromatic amino acid biosynthesis

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## ABSTRACT

Aromatic amino acids are precursors of numerous plant secondary metabolites with diverse biological functions. Many of these secondary metabolites are already being used as active pharmaceutical or nutraceutical ingredients, and there are numerous exploratory studies of other compounds with promising applications. *p*-Coumaric acid is derived from aromatic amino acids and, besides being a valuable chemical building block, it serves as precursor for biosynthesis of many secondary metabolites, such as polyphenols, flavonoids, and some polyketides.

Here we developed a *p*-coumaric acid-overproducing *Saccharomyces cerevisiae* platform strain. First, we reduced by-product formation by knocking out phenylpyruvate decarboxylase *ARO10* and pyruvate decarboxylase *PDC5*. Second, different versions of feedback-resistant DAHP synthase and chorismate mutase were overexpressed. Finally, we identified shikimate kinase as another important flux-controlling step in the aromatic amino acid pathway by overexpressing enzymes from *Escherichia coli*, homologous to the pentafunctional enzyme Aro1p and to the bifunctional chorismate synthase-flavin reductase Aro2p. The highest titer of *p*-coumaric acid of  $1.93 \pm 0.26 \text{ g L}^{-1}$  was obtained, when overexpressing tyrosine ammonia-lyase *TAL* from *Flavobacterium johnsoniae*, DAHP synthase *ARO4*<sup>K229L</sup>, chorismate mutase *ARO7*<sup>G141S</sup> and *E. coli* shikimate kinase II (*aroL*) in  $\Delta pdc5\Delta aro10$  strain background. To our knowledge this is the highest reported titer of an aromatic compound produced by yeast.

The developed *S. cerevisiae* strain represents an attractive platform host for production of *p*-coumaric-acid derived secondary metabolites, such as flavonoids, polyphenols, and polyketides.

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## 1. Introduction

Aromatic amino acids are precursors of many secondary metabolites produced in plants, where they have a key role in the plant development, adaptation and defense mechanisms (Maeda and Dudareva, 2012; Perez-Gregorio et al., 2014). Among the plant secondary metabolites many alkaloids, flavonoids, tannins and lignins find applications as nutraceutical and pharmaceutical ingredients (Scotti, 2012). Exploratory research of this type of compounds shows promising results (Winkel-Shirley,

2001; Hawkins and Smolke, 2008; Bhan et al., 2013; Leonard et al., 2009), but the limiting factor for a wider use of these secondary metabolites is the lack of efficient extraction systems from plants, or a competent microbial biosynthetic alternative that can produce these compounds in high yields (Santos et al., 2011). There is therefore much interest in developing a microbial cell factory platform that can be used for production of secondary metabolites derived from aromatic amino acids. As the biosynthesis of many plant secondary metabolites involves P450 enzymes, which are often difficult to express in bacteria, the yeast *Saccharomyces cerevisiae* is well suited as a cell factory platform for production of these products. Since many of the flavonoids are intended for nutraceutical applications, using *S. cerevisiae* as the host may be a further advantage due to the long history of its application in food and beverage production (Krivoruchko et al.,

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2011; Siddiqui et al., 2012) Furthermore, *S. cerevisiae* has proven well amenable for genetic engineering and industrial-scale fermentation and is currently used for production of active pharmaceutical ingredients, dietary supplements, chemicals and fuels (Hong and Nielsen, 2012; Nielsen et al., 2013; Borodina and Nielsen, 2014; Li and Borodina, 2015; Borodina et al., 2015).

In order to enable efficient production of secondary metabolites derived from aromatic amino acids it is necessary to optimize aromatic amino acid biosynthesis. *S. cerevisiae* has been metabolically engineered for improved production of aromatic amino acids through the elimination of feedback inhibition of key enzymes and elimination of by-product formation. Luttik et al. (2008) explored the use of mutated DAHP synthase *ARO4* and chorismate mutase *ARO7* to avoid feedback inhibition and got an increment of 200-fold of aromatic compounds in comparison to the reference strain. Furthermore, through expression of heterologous pathways, it has been possible to produce secondary metabolites derived from aromatic amino acids. Thus, Koopman et al. (2012) produced the flavonoid naringenin from glucose using a background strain with a triple knockout of the most active phenylpyruvate decarboxylases, which prevented formation of the by-product phenylethanol, and overexpressing feedback-resistant DAHP synthase and chorismate mutase.

*Escherichia coli* and *S. cerevisiae* have been extensively engineered in order to obtain flavonoid-producing cell factories. Among the flavonoids produced in *E. coli* from glucose are naringenin, pinocembrin, and kaempferol 3-O-rhamnoside, with titers of 84 mg L<sup>-1</sup>, 40 mg L<sup>-1</sup>, and 57 mg L<sup>-1</sup>, respectively (Santos et al., 2011; Wu et al., 2014; Yang et al., 2014). Several other flavonoids were produced by supplementing the broth with intermediate compounds, e.g., pinocembrin (429 mg L<sup>-1</sup>), naringenin (119 mg L<sup>-1</sup>), eriodictyol (52 mg L<sup>-1</sup>), quercetin (23.78 mg L<sup>-1</sup>), and resveratrol (2.3 g L<sup>-1</sup>) (Leonard et al., 2007; Pandey and Sohng, 2013; Lim et al., 2011). Flavonoids, such as resveratrol (0.31 mg L<sup>-1</sup>), genistein (7.7 mg L<sup>-1</sup>), kaempferol (4.6 mg L<sup>-1</sup>), and quercetin (0.38 mg L<sup>-1</sup>), have been produced by engineered *S. cerevisiae*, when supplemented with naringenin (Trantas et al., 2009). Using glucose as carbon source, Koopman et al. (2012) produced 102 mg L<sup>-1</sup> of naringenin. Jendresen et al. (2015) has reported several novel highly active tyrosine ammonia-lyases and shown their activity in *E. coli*, *Lactococcus lactis* and *S. cerevisiae*. These examples prove the potential of *E. coli* and *S. cerevisiae* for flavonoids production, but also evidence the need for development of a platform strain capable of high-level production of aromatic metabolites, e.g. *p*-Coumaric acid from which many secondary metabolites are derived (Santos et al., 2011). We therefore here developed a *S. cerevisiae* strain that overproduces *p*-coumaric acid, and besides representing a starting point for further development of a process for commercial *p*-coumaric acid, we believe this strain can be used as a platform strain for production of flavonoids and other coumaric-acid derived secondary metabolites.

## 2. Materials and methods

### 2.1. Plasmids and strains construction

The background strain for this research was *S. cerevisiae* CEN. PK102-5B. Cloning was carried out using *E. coli* strain DH5 $\alpha$ . All the fragments used for overexpression of genes were amplified by PCR using primers and templates as described in Supplementary Tables 1 and 2. The fragment encoding chorismate mutase from *C. guilliermondii* was identified through BLAST search, by comparing the full amino acids sequence of Aro7p from *S. cerevisiae* against *C. guilliermondii* protein sequences in GenBank. Homology of 60% was found between Aro7p from *S. cerevisiae* and the hypothetical

protein PGUG\_00476 from *C. guilliermondii* (Genbank accession number: XM\_001487049.1).

Tyrosine ammonia-lyase *TAL* from *F. johnsoniae* was as described before (Jendresen et al., 2015). The amplified products were cloned along with strong constitutive promoters into Easy-Clone integrative plasmids by USER cloning (Jensen et al., 2014). The clones with correct inserts were confirmed by sequencing. The list of the constructed vectors can be found in Table 1 and the details on the cloning are given in Supplementary Table 3.

Transformation of yeast cells was carried out by the lithium acetate method (Gietz et al., 2002). The strains were selected on synthetic drop-out medium (Sigma-Aldrich), selecting for *URA*, *HIS* and *LEU* markers. The yeast strains constructed in this study are listed in Table 1.

The mutant genes, *scARO4*<sup>fbt</sup> (K229L), *scARO7*<sup>fbt</sup> (G141S), *ecaroG*<sup>fbt1</sup> (L175D) and *ecaroG*<sup>fbt2</sup> (S180F), were constructed by site-directed mutagenesis method (Zheng et al., 2004), using primers and templates described in Supplementary Tables 1 and 3. The *ARO4*, *ARO7* and *aroG* wild-type genes were amplified from the genomic DNA of *S. cerevisiae* and *E. coli* NST 74. The DNA fragments were gel-purified and cloned into vector pESC-*URA*-ccdB-USER or pESC-*HIS*-ccdB-USER, the derived plasmids pCfB761, pCfB775, pCfB1075 and pCfB1076 were confirmed by DNA sequencing. These plasmids were used as the templates for site-directed mutagenesis reactions. The complementary primers with nucleotide substitutions for mutagenesis were designed for each mutation according to the guidelines stated by Zheng et al., 2004. The reactions were incubated with DpnI for 1 h before transformation into competent DH5 $\alpha$  *E. coli* cells, the strains were grown overnight on LB agar (Amp) plates at 37 °C. Colonies were then selected, and plasmid DNA was extracted and sequenced over the region of the mutation. Successful mutants were verified by DNA sequencing and then re-cloned into the integrative expression vectors (Supplementary Tables 2 and 3).

### 2.2. Deletions of *ARO10* and *PDC5*

The double knockout strain  $\Delta$ *aro10* $\Delta$ *pdcs* was constructed by an iterative replacement of the targeted genes with the *URA3* cassette in the strain CEN.PK102-5B by bi-partite method (Erdeniz et al., 1997). The knockout fragments were transformed into *S. cerevisiae* and transformants were selected on SC-*Ura* yeast synthetic drop-out media. The knockouts were confirmed by PCR on genomic DNA preparations. The *URA3* marker was looped-out via direct repeats by growing the yeast on 5-fluoroorotic acid (5-FOA) plates, and the second gene was knocked-out in the same way and the *URA3* marker was removed again.

For the single knockout strains ( $\Delta$ *aro10* and  $\Delta$ *pdcs*), the target genes were replaced by a *LEU2* cassette in the strain CEN.PK102-5B. The knockout fragments were transformed into *S. cerevisiae* and transformants were selected on SC-*Leu* yeast synthetic drop-out media. The knockouts were confirmed by PCR on genomic DNA preparations.

The gene fragments, carrying the upstream and downstream fragments of the marker cassettes *URA3* and *LEU2* and of the targeted genes *ARO10* and *PDC5*, were generated by PCR amplification using the method developed by Reid et al. (2002). Primers and templates used for targeting the genes are indicated in Supplementary Tables 1 and 2. The upstream fragments of the targeted genes (*PDC5*\_UP or *ARO10*\_UP), the downstream fragments of the targeted genes (*PDC5*\_DOWN or *ARO10*\_DOWN), the upstream and downstream fragment of the markers (2/3\_*URA3*\_UP, 2/3\_*URA3*\_DOWN, for the double knockout strain and 2/3\_*LEU2*\_UP and 2/3\_*LEU2*\_DOWN for the single knockout strain construction) from *Cluyveromyces lactis* were amplified using primers described in Supplementary Table 1. To generate a

**Table 1**  
Plasmids and strains used in this study.

Plasmid ID	Genotype	Source
<b>Parental plasmids</b>		
pCfB0054	Episomal replication vector, pESC, <i>HIS</i> , $P_{TEF1}$ - $T_{ADH1}$ , $P_{PGK1}$ - $T_{CYC1}$	Jensen et al. (2014)
pCfB0055	Episomal replication vector, pESC, <i>HIS</i> , $P_{TEF1}$ - $T_{ADH1}$ , $P_{PGK1}$ - $T_{CYC1}$	Jensen et al. (2014)
pCfB255	Integrative plasmid, pX-2-loxP, <i>klURA3</i> , $P_{TEF1}$ - $T_{ADH1}$ , $P_{PGK1}$ - $T_{CYC1}$	Jensen et al. (2014)
pCfB257	Integrative plasmid, pX-3-loxP, <i>klLEU2</i> , $P_{TEF1}$ - $T_{ADH1}$ , $P_{PGK1}$ - $P_{PGK1}$	Jensen et al. (2014)
pCfB258	Integrative plasmid, pX-4-loxP, <i>spHIS5</i> , $P_{TEF1}$ - $T_{ADH1}$ , $P_{PGK1}$ - $T_{CYC1}$	Jensen et al. (2014)
<b>Plasmids used for directed mutagenesis</b>		
pCfB744	Episomal replication vector, pESC, <i>URA</i> , $P_{TEF1}$ - <i>scARO7</i> - $T_{ADH1}$	This study
pCfB745	Episomal replication vector, pESC, <i>HIS</i> , $P_{TEF1}$ - <i>scARO4</i> - $T_{ADH1}$	This study
pCfB761	Episomal replication vector, pESC, <i>URA</i> , $P_{TEF1}$ - <i>scARO7<sup>br</sup></i> - $T_{ADH1}$	This study
pCfB775	Episomal replication vector, pESC, <i>HIS</i> , $P_{TEF1}$ - <i>scARO4<sup>br</sup></i> - $T_{ADH1}$	This study
PCfB1074	Integrative plasmid, pX-3-loxP, <i>klLEU2</i> , $P_{TEF1}$ - <i>ecaroG</i> - $T_{ADH1}$	This study
PCfB1075	Integrative plasmid, pX-3-loxP, <i>klLEU2</i> , $P_{TEF1}$ - <i>ecaroG<sup>br1</sup></i> - $T_{ADH1}$	This study
PCfB1076	Integrative plasmid, pX-3-loxP, <i>klLEU2</i> , $P_{TEF1}$ - <i>ecaroG<sup>br2</sup></i> - $T_{ADH1}$	This study
<b>Integrative plasmids</b>		
pCfB826	Integrative plasmid, pX-4-loxP, <i>spHIS5</i> , $P_{TEF1}$ - <i>scARO7<sup>br</sup></i> - $T_{ADH1}$ , $P_{PGK1}$ - <i>scARO4<sup>br</sup></i> - $T_{CYC1}$	This study
pCfB827	Integrative plasmid, pX-4-loxP, <i>spHIS5</i> , $P_{TEF1}$ - <i>scARO7<sup>br</sup></i> - $T_{ADH1}$ , $P_{PGK1}$ - <i>ecaroF<sup>br</sup></i> - $T_{CYC1}$	This study
pCfB830	Integrative plasmid, pX-4-loxP, <i>spHIS5</i> , $P_{TEF1}$ - <i>cgARO7</i> - $T_{ADH1}$ , $P_{PGK1}$ - <i>scARO4<sup>br</sup></i> - $T_{CYC1}$	This study
pCfB831	Integrative plasmid, pX-4-loxP, <i>spHIS5</i> , $P_{TEF1}$ - <i>cgARO7</i> - $T_{ADH1}$ , $P_{PGK1}$ - <i>ecaroF<sup>br</sup></i> - $T_{CYC1}$	This study
pCfB1077	Integrative plasmid, pX-4-loxP, <i>spHIS5</i> , $P_{TEF1}$ - <i>scARO7<sup>br</sup></i> - $T_{ADH1}$ , $P_{PGK1}$ - <i>ecaroG<sup>br1</sup></i> - $T_{CYC1}$	This study
pCfB1078	Integrative plasmid, pX-4-loxP, <i>spHIS5</i> , $P_{TEF1}$ - <i>cgARO7</i> - $T_{ADH1}$ , $P_{PGK1}$ - <i>ecaroG<sup>br1</sup></i> - $T_{CYC1}$	This study
pCfB1080	Integrative plasmid, pX-4-loxP, <i>spHIS5</i> , $P_{TEF1}$ - <i>scARO7<sup>br</sup></i> - $T_{ADH1}$ , $P_{PGK1}$ - <i>ecaroG<sup>br2</sup></i> - $T_{CYC1}$	This study
pCfB1081	Integrative plasmid, pX-4-loxP, <i>spHIS5</i> , $P_{TEF1}$ - <i>cgARO7</i> - $T_{ADH1}$ , $P_{PGK1}$ - <i>ecaroG<sup>br2</sup></i> - $T_{CYC1}$	This study
pCfB1221	Integrative plasmid, pX-3-loxP, <i>klLEU2</i> , $P_{TEF1}$ - <i>scTYR1</i> - $T_{ADH1}$	This study
pCfB1226	Integrative plasmid, pX-4-loxP, <i>spHIS5</i> , $P_{TEF1}$ - <i>ectyrA<sup>br</sup></i> - $T_{ADH1}$ , $P_{PGK1}$ - <i>scARO4<sup>br</sup></i> - $T_{CYC1}$	This study
pCfB1227	Integrative plasmid, pX-4-loxP, <i>spHIS5</i> , $P_{TEF1}$ - <i>ectyrA<sup>br</sup></i> - $T_{ADH1}$ , $P_{PGK1}$ - <i>ecaroF<sup>br</sup></i> - $T_{CYC1}$	This study
pCfB1228	Integrative plasmid, pX-4-loxP, <i>spHIS5</i> , $P_{TEF1}$ - <i>ectyrA<sup>br</sup></i> - $T_{ADH1}$ , $P_{PGK1}$ - <i>ecaroG<sup>br1</sup></i> - $T_{CYC1}$	This study
pCfB1964	Integrative plasmid, pX-2-loxP, <i>klURA3</i> , $P_{TEF1}$ - <i>fjTAL</i> - $T_{ADH1}$	This study
pCfB2733	Integrative plasmid, pX-3-loxP, <i>klLEU2</i> , $P_{TEF1}$ - <i>scARO1</i> - $T_{ADH1}$ , $P_{PGK1}$ - <i>scARO2</i> - $T_{CYC1}$	This study
pCfB2739	Integrative plasmid, pX-3-loxP, <i>klLEU2</i> , $P_{TEF1}$ - <i>ecaroB</i> - $T_{ADH1}$	This study
pCfB2741	Integrative plasmid, pX-3-loxP, <i>klLEU2</i> , $P_{TEF1}$ - <i>ecaroE</i> - $T_{ADH1}$	This study
pCfB2742	Integrative plasmid, pX-3-loxP, <i>klLEU2</i> , $P_{TEF1}$ - <i>ecydiB</i> - $T_{ADH1}$	This study
pCfB2743	Integrative plasmid, pX-3-loxP, <i>klLEU2</i> , $P_{PGK1}$ - <i>ecaroK</i> - $T_{CYC1}$	This study
pCfB2745	Integrative plasmid, pX-3-loxP, <i>klLEU2</i> , $P_{TEF1}$ - <i>ecaroA</i> - $T_{ADH1}$	This study
pCfB2746	Integrative plasmid, pX-3-loxP, <i>klLEU2</i> , $P_{PGK1}$ - <i>ecaroD</i> - $T_{CYC1}$	This study
pCfB2747	Integrative plasmid, pX-3-loxP, <i>klLEU2</i> , $P_{PGK1}$ - <i>ecaroL</i> - $T_{CYC1}$	This study
pCfB2749	Integrative plasmid, pX-3-loxP, <i>klLEU2</i> , $P_{PGK1}$ - <i>ecaroC</i> - $T_{CYC1}$	This study
pCfB2740	Integrative plasmid, pX-3-loxP, <i>klLEU2</i> , $P_{TEF1}$ - <i>scARO1</i> - $T_{ADH1}$	This study
pCfB2748	Integrative plasmid, pX-3-loxP, <i>klLEU2</i> , $P_{PGK1}$ - <i>scARO2</i> - $T_{CYC1}$	This study
<b>Parent and template strains</b>		
<b>Strain ID</b>	<b>Strain</b>	<b>Source</b>
ST10	<i>S. cerevisiae</i> CEN.PK102-5B (MATa <i>ura3-52 his3 Δ 1 leu2-3/112 MAL2-8c SUC2</i> )	Peter Kötter
ST700	<i>E. coli</i> NST 74 (ATCC 31884)	ATCC
ST679	<i>C. guilliermondii</i> (ATCC 6260)	ATCC
<b>Knockout strains</b>		
<b>Strain ID</b>	<b>Parent strain</b>	<b>Characteristics</b>
ST4034	ST10	MATa <i>aro10Δ: LEU2</i>
ST3532	ST10	MATa <i>pdv5Δ: LEU2</i>
ST691	ST10	MATa <i>aro10Δ pdv5Δ</i>
<b>Strains transformed with integrative plasmids</b>		
<b>Strain ID</b>	<b>Parent strain</b>	<b>Integrated plasmids</b>
ST4068	ST10	pCfB1964, pCfB257, pCfB258
ST4069	ST10	pCfB255, pCfB257, pCfB258

Table 1 (continued)

Plasmid ID	Genotype		Source
ST4072	ST4034	pCfB1964, pCfB258	This study
ST4073	ST4034	pCfB255, pCfB258	This study
ST4070	ST3532	pCfB1964	This study
ST4071	ST3532	pCfB255	This study
ST4048	ST691	pCfB1964, pCfB257, pCfB258	This study
ST4050	ST691	pCfB255, pCfB257, pCfB258	This study
ST2645	ST691	pCfB1964, pCfB257, pCfB826	This study
ST4040	ST691	pCfB1964, pCfB1221, pCfB826	This study
ST4049	ST691	pCfB1964, pCfB257, pCfB830	This study
ST4044	ST691	pCfB1964, pCfB1221, pCfB830	This study
ST4038	ST691	pCfB1964, pCfB257, pCfB1226	This study
ST4041	ST691	pCfB1964, pCfB1221, pCfB827	This study
ST4051	ST691	pCfB1964, pCfB257, pCfB827	This study
ST4045	ST691	pCfB1964, pCfB1221, pCfB831	This study
ST4052	ST691	pCfB1964, pCfB257, pCfB831	This study
ST4037	ST691	pCfB1964, pCfB257, pCfB1227	This study
ST4053	ST691	pCfB1964, pCfB257, pCfB1077	This study
ST4042	ST691	pCfB1964, pCfB1221, pCfB1077	This study
ST4054	ST691	pCfB1964, pCfB257, pCfB1078	This study
ST4046	ST691	pCfB1964, pCfB1221, pCfB1078	This study
ST4039	ST691	pCfB1964, pCfB257, pCfB1228	This study
ST4055	ST691	pCfB1964, pCfB257, pCfB1080	This study
ST4043	ST691	pCfB1964, pCfB1221, pCfB1080	This study
ST4056	ST691	pCfB1964, pCfB257, pCfB1081	This study
ST4047	ST691	pCfB1964, pCfB1221, pCfB1081	This study
ST3213	ST691	pCfB1964, pCfB826	This study
ST4057	ST3213	pCfB2739	This study
ST4065	ST3213	pCfB2746	This study
ST4062	ST3213	pCfB2741	This study
ST4063	ST3213	pCfB2742	This study
ST4066	ST3213	pCfB2743	This study
ST4058	ST3213	pCfB2747	This study
ST4064	ST3213	pCfB2745	This study
ST4060	ST3213	pCfB2749	This study
ST4067	ST3213	pCfB2733	This study
ST4061	ST3213	pCfB2740	This study
ST4059	ST3213	pCfB2748	This study

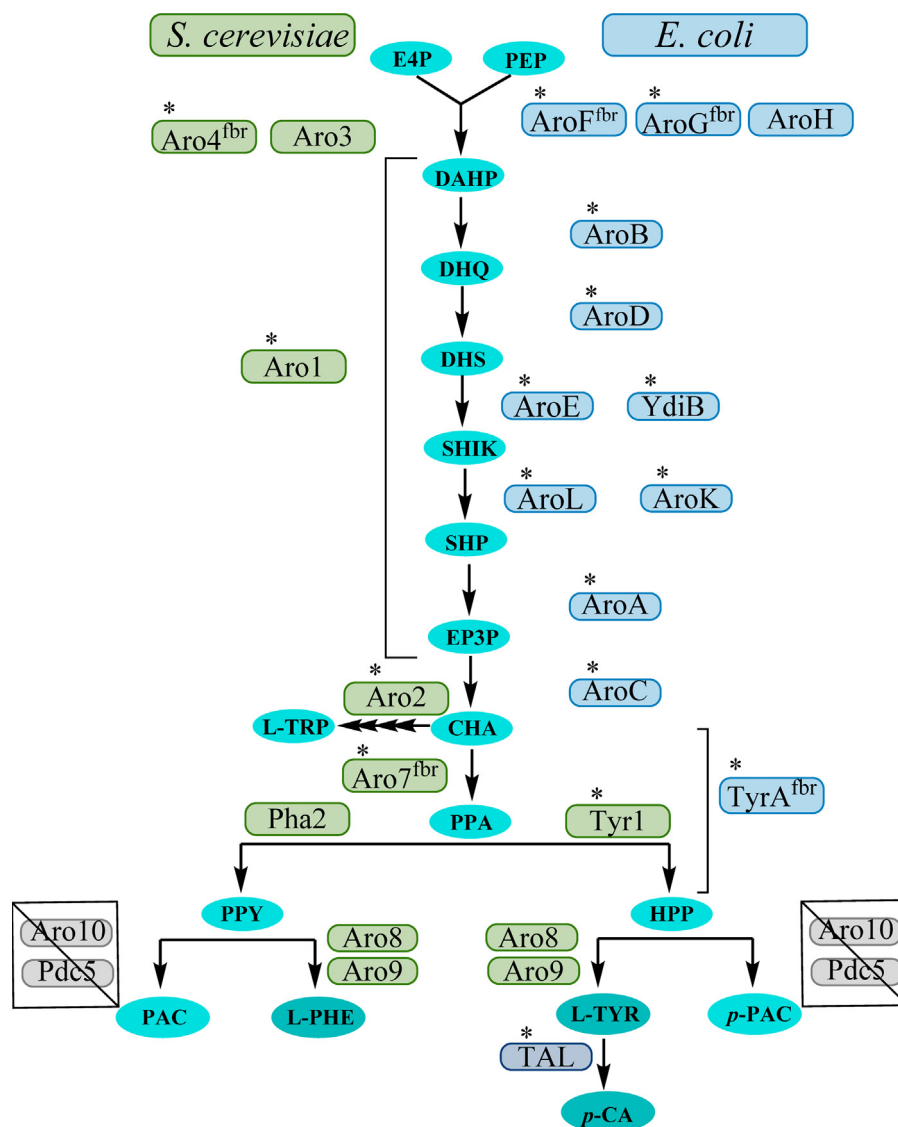
complete gene targeting substrate, the upstream fragments (*PDC5\_UP* or *ARO10\_UP*), were fused to the 2/3 upstream fragment of the markers (2/3\_URA3\_UP for the double knockout and 2/3\_LEU2\_UP for the single knockout), in the same way, the downstream fragments were fused to the downstream fragment of the markers (2/3\_URA3\_DOWN for the double knockout and 2/3\_LEU2\_DOWN for the single knockout). The two fusion PCR fragments per targeted gene were transformed simultaneously into the *S. cerevisiae* strain and selected in SC-Ura or SC-Leu medium according to the selection marker. The correct transformants were confirmed by PCR, using primers described in Supplementary Table 1.

### 2.3. Media and cultivations

Synthetic complete (SC) medium as well as drop-out media (SC-Ura, SC-Leu, SC-His) and agar plates were prepared using pre-mixed drop-out powders from Sigma-Aldrich. Synthetic fed-batch medium for *S. cerevisiae* M-Sc.syn-1000 (FIT) was purchased from M2P labs GmbH (Germany). The medium was supplemented with the supplied vitamins solution (final 1% v/v) and the enzyme mix (final concentration 0.5% v/v) immediately prior to use.

At least six single colonies originating from independent transformants were inoculated in 0.5 ml drop-out SC liquid medium without uracil, histidine, and/or leucine in 96-deep well





**Fig. 1.** Schematic representation of the engineered *p*-coumaric acid production pathway in *S. cerevisiae*. E4P: erythrose 4-phosphate, PEP: phosphoenolpyruvate, DAHP: 3-deoxy-D-arabino-heptulosonic acid 7-phosphate, DHQ: 3-dehydroquinone, DHS: 3-dehydro-shikimate, SHIK: shikimate, SHP: shikimate-3-phosphate, EP3P: 5-enolpyruvylshikimate-3-phosphate, PPA: prephenate, PPY: phenylpyruvate, HPP: para-hydroxy-phenylpyruvate, PAC: phenylacetaldehyde, pPAC: para-hydroxy-acetaldehyde, L-PHE: L-phenylalanine, L-TYR: L-tyrosine, *p*-CA: *p*-coumaric acid, TAL: tyrosine ammonia-lyase. The star key indicates overexpressed enzymes; enzymes in gray boxes represent knockouts, "fbr" indicates feedback-resistant.

microtiter plates with air-penetrable lid (EnzyScreen, NL). The plates were incubated at 30 °C with 250 rpm agitation at 5 cm orbit cast overnight. 50 µl of the overnight cultures were used to inoculate 0.5 ml synthetic fed-batch medium in a 96-deep well plate. Fermentation was carried out for 72 h at the same conditions as above.

At the end of the cultivation OD<sub>600</sub> was measured as following: 10 µl of the sample was mixed with 190 µl water and absorbance was measured at 600 nm wavelength in microplate reader BioTek Synergy MX (BioTek). The culture broth was spun down and the supernatant was analyzed for *p*-coumaric acid concentration using HPLC.

#### 2.4. Quantification of *p*-coumaric acid

Quantification of *p*-coumaric acid was performed on HPLC (Thermo), equipped with a Discovery HS F5 150 mm × 2.1 mm column (particle size 3 µm). Samples were analyzed using a gradient method with two solvents: 10 mM ammonium formate pH 3.0 (A) and acetonitrile (B) at 1.5 ml min<sup>-1</sup>. The program

started with 5% of solvent B (0–0.5 min), after which its fraction was increased linearly from 5% to 60% (0.5–7.0 min) and maintained at 60% for 2.5 min (7.0–9.5 min). Then the fraction of solvent B was decreased back to 5% (9.5–9.6 min) and remained at 5% until the end (9.6–12 min). *p*-Coumaric acid was detected by absorbance at 277 nm and the peak (retention time 4.7 min) area was integrated with Chromeleon 7 and used for quantification by fitting with a standard curve. For all the strains at least three biological replicates were analyzed.

### 3. Results

#### 3.1. Deletion of phenylpyruvate and pyruvate decarboxylases

In order to avoid production of aromatic alcohols and direct the pathway flux to aromatic amino acids, we performed single knockouts of *ARO10* (phenylpyruvate decarboxylase), *PDC5* (pyruvate decarboxylase), and a double knock out of *ARO10* and *PDC5*.

Furthermore, a tyrosine ammonia-lyase TAL from *F. johnsoniae* was overexpressed in these strains in order to produce *p*-coumaric acid (Jensen et al., 2014) (Fig. 1).

The reference strain without deletions was able to produce  $0.24 \pm 0.03 \text{ g L}^{-1}$  of *p*-coumaric acid (Fig. 2). The strain with the single deletion of *PDC5* produced  $0.30 \pm 0.09 \text{ g L}^{-1}$  of *p*-coumaric acid, while the strain carrying the knockout of *ARO10* produced  $0.29 \pm 0.04 \text{ g L}^{-1}$ . The highest production ( $0.55 \pm 0.13 \text{ g L}^{-1}$ ) was obtained in the strain with the double knockout of *ARO10* and *PDC5*.

### 3.2. Effect of the elimination of the feedback inhibition of DAHP synthase and chorismate mutase on *p*-coumaric acid production

The enzymes DAHP synthase and chorismate mutase from the aromatic amino acids pathway are feedback-inhibited by L-tyrosine and L-phenylalanine (Hartmann et al., 2003; Luttk et al., 2008). In order to enhance the activity of these enzymes, we overexpressed feedback-resistant variants of DAHP synthase and chorismate mutase in the  $\Delta\text{aro10}\Delta\text{pdc5}$  strain. For this we selected 4 variants of DAHP synthase, a mutated feedback-resistant *ARO4*<sup>K229L</sup> from *S. cerevisiae*, an *aroF* from *E. coli* NST 74 (ATCC 31884) and two mutant variants of *aroG*, also from *E. coli*, which were constructed through replacement of the residues L175D and S180F of the hydrophobic domain. For chorismate mutase, there were selected a mutated feedback-resistant *ARO7*<sup>G141S</sup> from *S. cerevisiae*, a naturally feedback-resistant chorismate mutase from *C. guilliermondii* and *tyrA* from the *E. coli* strain NST 74 (ATCC 31884) (Tribe, 1987). The chorismate mutase from *C.*

*guilliermondii* had been reported as non-feedback inhibited (Bode and Birnbaum, 1991). The variants of chorismate mutase from *S. cerevisiae* and *C. guilliermondii* were complemented with prephenate dehydrogenase *TYR1* from *S. cerevisiae* in order to get equivalent overexpressions to the bifunctional chorismate mutase-prephenate dehydrogenase *TyrA* from *E. coli* NST 74 (ATCC 31884). The mutations were selected from previous studies, where they had been reported as feedback-insensitive mutations: the mutations in *ARO4* and *ARO7* were reported by Luttk et al. (2008), the mutation of *aroG* (L175D) was reported by Hu et al. (2003) and the mutation of *aroG* (S180F) was reported by Ger et al. (1994).

All the strains overexpressing DAHP synthase and chorismate mutase were evaluated for their ability to produce *p*-coumaric acid and a two-way ANOVA was conducted to analyze the effect of DAHP synthases and chorismate mutases. The *p*-coumaric acid production was normally distributed for all the combinations of DAHP synthases and chorismate mutases as assessed by a Shapiro–Wilk's test (*p*-Value > 0.05). The results showed that the overexpression of chorismate mutase alone did not have a significant effect on the production of *p*-coumaric acid (*p*-Value 0.399), while the overexpression of DAHP synthase and the combined overexpression of DAHP synthase and chorismate mutase had a significant effect on the production of the compound (*p*-Values 0.010 and 0.0005 correspondingly) (Supplementary Tables 4 and 5).

From the strains overexpressing *scARO4*<sup>br</sup>, the best combination was obtained, when overexpressing at the same time *scARO7*<sup>br</sup>, for the strains overexpressing *ecaroF* the best producer was obtained in combination with *cgARO7*<sup>br</sup>. Although the two residues replaced in the *aroG* strains are located in the same region, the *p*-coumaric acid production after the overexpression of the mutated *AroG* enzymes was different. The replacement L175D seems to generate a more active *AroG*, since two of the strains carrying this mutation (*ecaroG*<sup>br1</sup>-*ectyrA* and *ecaroG*<sup>br1</sup>-*cgARO7*) had production of over  $0.9 \text{ g L}^{-1}$  in contrast to the strains with the replacement S180F, where the titer did not exceed  $0.8 \text{ g L}^{-1}$ . It was not possible to see a general trend of the effect of *Tyr1p* in the production of *p*-coumaric acid, but in 4 of the 8 strains overexpressing this enzyme a negative effect was observed (Fig. 3).

The overexpression of DAHP synthase and chorismate mutase mostly had a positive effect on production of *p*-coumaric acid, however there were some exceptions: *scARO4*-*cgARO7*, *scARO4*-*ectyrA*, *ecaroG*-*ARO7* and *ecaroG*-*scARO7*-*scTYR1* all resulted in the same or lower titer than in the reference strain.

### 3.3. *p*-Coumaric acid production after overexpression of *ARO1* and *ARO2* from *S. cerevisiae* and their analogous from *E. coli*

In *S. cerevisiae*, the five steps to synthesize the aromatic intermediate compound 5-enolpyruvylshikimate-3-phosphate (EPSP) from DAHP are catalyzed by the pentafunctional enzyme *Aro1p*, while in other organisms such as plants and bacteria each step is performed by monofunctional enzymes (Fig. 1). In order to find flux-controlling steps in this common branch of the aromatic amino acid pathway, analogous enzymes to *Aro1p* and *Aro2p* from *E. coli* were overexpressed in *S. cerevisiae*. Strains overexpressing *ARO1* and *ARO2* from *S. cerevisiae* were also constructed with the purpose of making a comparative analysis between the strains overexpressing enzymes from *E. coli* and *S. cerevisiae*. The background strain for this experiment was the strain ST3213 (*aro10* $\Delta$ *pdc5* $\Delta$  *ARO4*<sup>K229L</sup> *ARO7*<sup>G141S</sup>). The control strain for this experiment was the strain ST3213 transformed with the empty integrative plasmid pCB257 instead of plasmids carrying overexpression cassettes for *ARO1*, *ARO2* or their analogous.

The overexpression of the monofunctional enzymes from *E. coli* had a positive effect on the *p*-coumaric acid production; the only exception was the overexpression of shikimate kinase *AroK* (Fig. 4A).

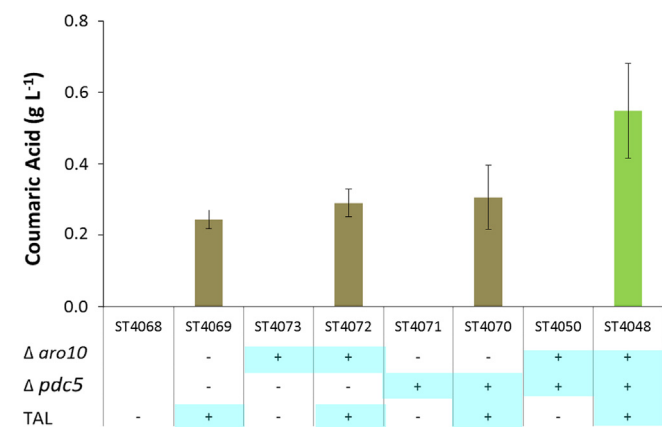


Fig. 2. Effect of knockouts of *ARO10* and *PDC5* on *p*-coumaric acid production in strains overexpressing tyrosine-ammonia lyase *FjTAL* from *F. johnsoniae*.

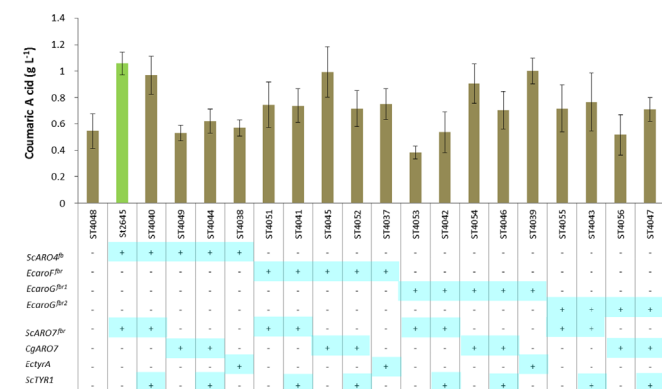


Fig. 3. Production of *p*-coumaric acid upon overexpression of feedback inhibition-resistant DAHP synthase and chorismate mutase in strains with overexpression of *FjTAL* and deletion of *PDC5* and *ARO10*.

The strains overexpressing dehydroquinase synthase (AroB), shikimate dehydrogenase (YdiB), EPSP synthase (AroA) or shikimate kinase (AroL) produced more than  $1.6 \text{ g L}^{-1}$  of *p*-coumaric acid. The strain with the highest improvement was the strain overexpressing the isoenzyme of shikimate kinase AroL, producing  $1.93 \pm 0.26 \text{ g L}^{-1}$  of *p*-coumaric acid.

Overexpression of the pentafunctional enzyme Aro1p and the bifunctional chorismate synthase-flavin reductase Aro2p from *S. cerevisiae* had a positive effect in the *p*-coumaric acid production. The strain overexpressing Aro1p produced  $1.68 \pm 0.19 \text{ g L}^{-1}$  of *p*-coumaric acid, the strain overexpressing Aro2p produced  $1.40 \pm 0.12 \text{ g L}^{-1}$ , and the simultaneous overexpression of Aro1p and Aro2p increased the production of *p*-coumaric acid to  $1.71 \pm 0.12 \text{ g L}^{-1}$ . The production of *p*-coumaric acid was very similar between the strain overexpressing Aro1p and the strain overexpressing Aro1p and Aro2p simultaneously. None of the strains expressing native versions of Aro1p and Aro2p produced more *p*-coumaric acid than the strain overexpressing AroL from *E. coli* (Fig. 4B).

#### 4. Discussion

This study describes engineering of *S. cerevisiae* for production of *p*-coumaric acid from glucose, leading to a final production titer of  $1.93 \pm 0.26 \text{ g L}^{-1}$  on feed-in-time medium in deep-well plates, which represents a 7.9-fold improvement in comparison to the non-optimized strain. The optimized strain also produced  $1.89 \text{ g L}^{-1}$  *p*-coumaric acid in controlled fed-batch fermentation on mineral medium (Supplementary Fig. 1). To the best of our knowledge, this is the highest titer of *de novo* production of an aromatic compound reported for *S. cerevisiae*. There are studies of flavonoids production from glucose in *E. coli* and *S. cerevisiae*;

however none of them has *p*-coumaric acid as the final product. Some studies reported the accumulation of *p*-coumaric acid in parallel to the production of other aromatic compounds. In *E. coli*, Santos et al. (2011) and Wu et al. (2014) reported accumulation of *p*-coumaric acid to 79 and  $70 \text{ mg L}^{-1}$ , respectively, when they were producing 84 and  $101 \text{ mg L}^{-1}$  of naringenin. In *S. cerevisiae*, Koopman et al. (2012) reported around  $61 \text{ mg L}^{-1}$  of accumulated *p*-coumaric acid, when they were producing  $65 \text{ mg L}^{-1}$  of naringenin in bioreactors.

The single knockouts of *PDC5* and *ARO10* had a positive effect on the production *p*-coumaric acid. The *PDC5* strain was auxotrophic for histidine, and the effect of the *PDC5* knockout in comparison to *ARO10* may be different in a prototrophic strain. The simultaneous deletions lead a 2-folds improvement in comparison to the reference strain (Fig. 2). This was expected as deletion of the two genes had been previously reported to improve production of some other tyrosine- and phenylalanine-derived compounds, i.e., naringenin (Koopman et al., 2012).

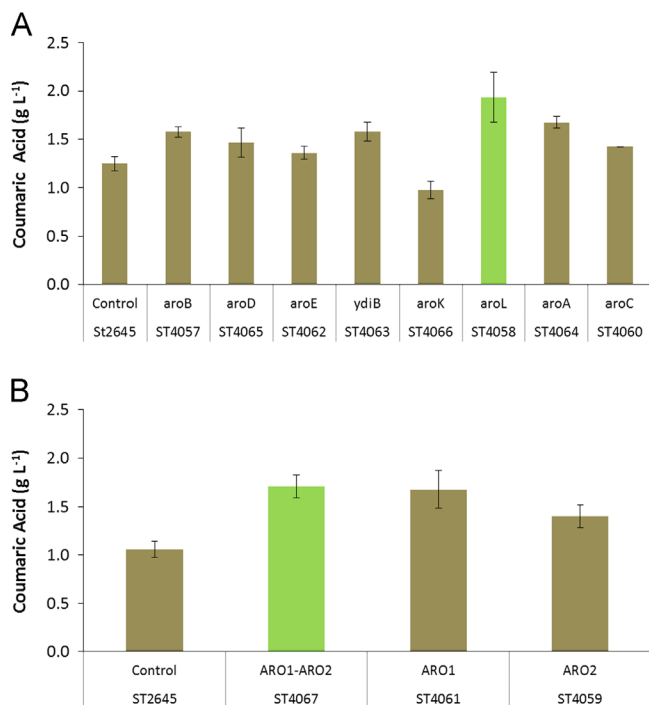
The results obtained from the 2-factor Anova showed that overexpression of chorismate mutase alone did not have a statistically significant effect on production of *p*-coumaric acid. This is consistent with results obtained by Luttik et al. (2008), where overexpression of a native or a feedback resistant Aro7 did not have significant impact on the production of the aromatic fusel alcohols, unless Aro4 was overexpressed as well.

Besides Aro4p and Aro7p that have been widely reported as feedback controlled enzymes, there must be other enzymes limiting the production of aromatic amino acids. To this end, we showed that the activity of the pentafunctional enzyme Aro1p in *S. cerevisiae* is also limiting for the biosynthesis of chorismate. With exception of the strain overexpressing AroK, all the strains overexpressing the Aro1p and Aro2p analogous enzymes from *E. coli* had a higher production of *p*-coumaric acid (Fig. 4A). These results show that these enzymes also exhibit flux control and further improvements can be obtained through the overexpression of heterologous enzymes with higher activity than Aro1p and Aro2p from *S. cerevisiae*.

The strain overexpressing AroL from *E. coli* had the highest production of *p*-coumaric acid, indicating that conversion of shikimate to shikimate-3-phosphate has the highest flux control of these five steps. These results are supported by the observation from Luttik et al. (2008), where they reported accumulation of shikimate in the culture supernatants after overexpression of scAro4<sup>fb</sup> in *S. cerevisiae*. Previous studies in *E. coli* also reported increased flux of intermediate compounds to L-tyrosine, when shikimate kinase II was overexpressed (Takai et al., 2005; Juminaga et al., 2012).

The overexpression of AroK did not increase the production of *p*-coumaric acid in the constructed strain. This may be due to the low affinity of AroK to shikimate, i.e. the  $K_m$  for shikimate of AroK is more than 20 mM whereas the  $K_m$  of AroL is only 0.2 mM (DeFeyter and Pittard, 1986). The contribution of AroK to the shikimate kinase activity in *E. coli* is therefore minimal (DeFeyter and Pittard, 1986) and its overexpression did not have a significant effect in *p*-coumaric acid production in *E. coli*. Previously there was not detected any feedback inhibition of AroL or AroK by aromatic amino acids, chorismic acid or prephenic acid (DeFeyter and Pittard, 1986). Overexpression of the pentafunctional protein (Aro1p) and the bifunctional chorismate synthase-flavin reductase (Aro2p) from *S. cerevisiae* had a positive effect on the production of *p*-coumaric acid, though the titer was still lower than of the strain overexpressing AroL alone.

The *p*-coumaric acid overproducing strain that we describe can be further engineered to include overproduction of malonyl-CoA, a common precursor for biosynthesis of polyphenols and flavonoids. It has been previously shown that increasing malonyl-CoA supply improves production of naringenin (Koopman et al., 2012),



**Fig. 4.** Production of *p*-coumaric acid upon overexpression of ARO1 and ARO2 from *S. cerevisiae* and their analogous from *E. coli* along with overexpression of ARO4<sup>K229L</sup>, ARO7<sup>G141S</sup>, FjTAL and knockouts of ARO10 and PDC5. (A) Genes from *E. coli*; aroB: 3-dehydroquinase synthase, aroD: 3-dehydroquinase dehydratase, aroE: shikimate dehydrogenase, ydiB: shikimate dehydrogenase – quinase dehydrogenase, aroK: shikimate kinase I, aroL: shikimate kinase II, aroA: EPSP synthase, aroC: chorismate synthase. (B) Genes from *S. cerevisiae*; ARO1: pentafunctional enzyme, ARO2: bifunctional chorismate synthase and flavin reductase. Control strain ST2645 (FjTAL, aro10Δpdc5Δ, ARO4<sup>K229L</sup>, ARO7<sup>G141S</sup>).



flavonone (Leonard et al., 2007), and 7-O-methyl aromadendrin (Malla et al., 2012). Increased flux towards malonyl-CoA can be achieved by overexpression of deregulated acetyl-CoA carboxylase (Shi et al., 2014) and by further increase of acetyl-CoA biosynthesis as described previously (Krivoruchko et al., 2015).

In conclusion, we describe metabolic engineering strategies that lead towards a platform yeast strain, producing high levels of *p*-coumaric acid, which besides being a product of commercial interest by itself, also serves as an intermediate compound for aromatic secondary metabolites. We also demonstrate that heterologous expression of tyrosine-ammonia lyase *TAL* is well suitable for evaluation of metabolic engineering targets for improving the flux through the aromatic amino acid biosynthetic pathway. Through combination of several different strategies we improved the production of *p*-coumaric acid 7.9-fold, and we are therefore confident that our strain represents a good platform strain for production of *p*-coumaric acid derived secondary metabolites by *S. cerevisiae*.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ymben.2015.08.003>.

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