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Comparison of the metabolic response to over-production of *p*-coumaric acid in two yeast strains

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ABSTRACT

The development of robust and efficient cell factories requires understanding of the metabolic changes triggered by the production of the targeted compound. Here we aimed to study how production of *p*-coumaric acid, a precursor of multiple secondary aromatic metabolites, influences the cellular metabolism of *Saccharomyces cerevisiae*. We evaluated the growth and *p*-coumaric acid production in batch and chemostat cultivations and analyzed the transcriptome and intracellular metabolome during steady state in low- and high-producers of *p*-coumaric acid in two strain backgrounds, S288c or CEN.PK.

We found that the same genetic modifications resulted in higher production of *p*-coumaric acid in the CEN.PK background than in the S288c background. Moreover, the CEN.PK strain was less affected by the genetic engineering as was evident from fewer changes in the transcription profile and intracellular metabolites concentrations. Surprisingly, for both strains we found the largest transcriptional changes in genes involved in transport of amino acids and sugars, which were downregulated. Additionally, in S288c amino acid and protein biosynthesis processes were also affected.

We systematically overexpressed or deleted genes with significant transcriptional changes in CEN.PK low and high-producing strains. The knockout of some of the downregulated transporters triggered a 20–50% improvement in the synthesis of *p*-CA in the CEN.PK high-producing strain. This study demonstrates the importance of transporters in the engineering of cell factories for production of small molecules.

1. Introduction

Plants produce a wide range of secondary metabolites as a protective mechanism to stresses caused by bacterial or viral infections, ultraviolet radiation, wounds, and other biotic and abiotic factors. Nearly 15% of these metabolites are phenolic compounds derived from the aromatic amino acids L-tyrosine, L-phenylalanine or L-tryptophan (Wink, 2010). Numerous aromatic secondary metabolites are available on the market as therapeutic agents, dyes, fragrances, and flavors. The majority of these compounds are currently synthesized chemically or isolated from plants (Bourgaud et al., 2001), however recently there have been significant advances in engineering industrial microbes, e.g., *Escherichia coli* and *S. cerevisiae*, for production of aromatic secondary metabolites by fermentation. A few biotech-derived aromatics are already on the market, such as phenylalanine, resveratrol, vanillin, steviol glucoside, and others. Additionally, many aromatic metabolites have been produced in microbial cell factories at proof-of-concept levels, but the strains, fermentation and downstream processes need further development before the production becomes economically feasible. These compounds include naringenin, genistein, kaempferol, fisetin, melatonin, and many others (Koopman et al., 2012; Trantas et al., 2009; Santos et al., 2011; Leonard et al., 2009; Stahlhut et al., 2015; Krivoruchko and Nielsen, 2015; Li et al., 2015; Germann et al., 2016).

An important step towards improved microbial cell factories is a better understanding of how the engineered cells respond to production of target compounds (Nielsen and Keasling, 2016). For this purpose, omic-level characterization of the strains is useful since the organism can be studied at different levels and the information can be assessed in

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the context of cellular metabolism (Kim et al., 2012; Curran and Alper, 2012). There are a few successful examples of applying systems biology for guiding metabolic engineering strategies. Otero et al. (2013) obtained a 30-fold improvement in succinic acid production in *S. cerevisiae* based on the integrative analysis of physiology and transcriptome data. Park et al. (2007) engineered an efficient L-valine-producing *E. coli* by using transcriptomic analysis together with *in silico* models. A multi-omic analysis of two different *E. coli* strains allowed Yoon et al. (2012) to identify an optimal strain for production of recombinant proteins. This study is one of the few that considered the differences between strains of the same species, when selecting the suitable host organism. In *S. cerevisiae*, a considerable number of differences have been found in the genomes of two widely used strains, CEN.PK and S288c. These differences are mainly related to the presence of 13,787 single nucleotide polymorphisms, 939 of them related to 158 genes involved in metabolic functions with enrichment in the galactose uptake and ergosterol biosynthetic pathways. Moreover, 83 genes, mainly located in sub-telomeric regions of S288c, are absent in the CEN.PK strain (Otero et al., 2010; Nijkamp et al., 2012).

The strain CEN.PK is widely used for industrial biotechnology research and applications, whereas the strain S288c is widely used in genetic studies. Recently the strain S288c has also been used for the production of some metabolites, such as vanillin-β-glucoside, 2-phenylethanol and methionol (Strucko et al., 2015; Yin et al., 2015a, 2015b). In the particular case of vanillin-β-glucoside, the engineered S288c strain produced 10-fold more product than the CEN.PK strain engineered in the same way and this effect was associated with several single nucleotide polymorphisms in the shikimate pathway genes.

p-Coumaric acid (*p*-CA) is a precursor for biosynthesis of a number of secondary metabolites, such as polyphenols, flavonoids, and some polyketides. We recently reported the engineering of *S. cerevisiae* as a cell factory for high-level production of *p*-CA with a titer of ~ 2 g/L (Rodriguez et al., 2015). In this study, we aimed to investigate firstly how the production of *p*-CA influences the host and secondly whether these effects depend on the strain background. To answer these two questions, we performed transcriptome and intracellular metabolome analysis on S288c and CEN.PK strains, which either only expressed an enzyme for making the product (low-producers) or were additionally optimized towards production of aromatic products (high-producers).

2. Materials and methods

2.1. Plasmids and strains

E. coli DH5*α* was used for cloning procedures. The fragments used for overexpression of genes were amplified by PCR using primers and templates as described in the Tables S1 and S2. The fragments were amplified from the genomic DNA of *S. cerevisiae* CEN.PK102-5B *(*MATa *ura*3-52 *his*3Δ1 *leu*2-3/112 MAL2-8c SUC2*)* and *E. coli* NST 74. The gene encoding tyrosine ammonia-lyase from *Flavobacterium johnsoniae* (FjTAL) was as described in (Jendresen et al., 2015). The amplified gene-encoding fragments were cloned together with strong constitutive promoters into EasyClone integrative plasmids by USER cloning (Jensen et al., 2014; Jessop-Fabre et al., 2016). The clones were tested for correct insertion of gene/promoter fragments by colony PCR using the primers summarized in the Table S1 and the resulting plasmids were verified by sequencing. The list of the constructed vectors can be found in Table 1.

S. cerevisiae CEN.PK113-7D was obtained from Peter Kötter (Johann Wolfgang Goethe-University Frankfurt, Germany). The strain BY4741, a derivative of strain S288c, was obtained from EUROSCARF. Transformation of yeast cells was performed using the lithium acetate method (Gietz et al., 2002). The strains were selected on synthetic drop-out medium (Sigma-Aldrich) and the genetic modifications were confirmed by colony PCR. The yeast strains used in this study are listed in Table 2 and Table S3.

2.2. Media and cultivations.

We prepared a mineral medium for the batch fermentation according to Verduyn et al. (1992). Glucose concentration in batch medium was 40 g l^{-1}. The feed medium for chemostats was prepared in the same way, but the amount of glucose was reduced to 10 g l^{-1} and the medium was supplemented with 0.2 ml L^{-1} of 2 M KOH and one drop of antifoam 204 (Sigma A-8311) per 20 L of medium. The pre-culture was done by inoculating a yeast colony into 50 ml of mineral medium in a 250-ml baffled shake flask and incubating the culture with shaking at 200 rpm at 30 °C for around 12 h. When the pre-culture reached OD_{600} of ca. 2, it was used to inoculate a bioreactor to a starting optical density of 0.05.

The fermentations were performed in DasGip 1-L stirrer-pro vessels (Eppendorf, Jülich, Germany), using the working volume of 500 ml. The temperature was 30 °C, agitation was at 600 rpm and aeration at 1 vvm. pH was monitored with a pH sensor (Mettler Toledo, Switzerland) and pH was maintained at 5.0 ± 0.05 by automatic addition of 2 M KOH. Dissolved oxygen was above 30% throughout the fermentation as measured by the polarographic oxygen sensor (Mettler Toledo, Greifensee, Switzerland). The completion of the batch phase was determined by monitoring CO_2 in the exhaust gas, when the second CO_2 peak, corresponding to ethanol consumption phase, declined. We then initiated constant feed to obtain glucose-limited steady-state with dilution rate of 0.100 ± 0.005 h^{-1}. The volume was kept constant using an overflow pump. The samples for transcriptome and metabolome analysis were taken after 4 residence times of steady-state growth. Four technical replicates were taken from each reactor for transcriptome and metabolome analyses. Each strain was fermented twice to obtain 2 biological replicates.

2.3. Analytical methods

For analysis of extracellular metabolites and the biomass, we withdrew ca. 3-ml samples from the reactor. 1 ml of the sample was centrifuged at 11,000*g* for 5 min and stored at −20 °C until HPLC analysis for glucose and organic acids. For *p*-CA analysis in the optimized strains (ST4288 and ST4353) we mixed 1 vol of sample with 9 volumes of 50% ethanol, whereas for the non-optimized strains (ST4408 and ST4397) we mixed 1 vol of sample with 1 vol of 50% ethanol. This was done to dissolve the *p*-CA that may have precipitated from the broth due to poor solubility in water. These samples were also centrifuged at 11,000 × *g* for 5 min and stored at −20 °C until further analysis.

The analysis of glucose, glycerol, ethanol, and organic acids was performed on Dionex Ultimate 3000 high-performance liquid chromatography (HPLC) system (Dionex Softron GmbH, Germany), with an Aminex HPX-87H column (Bio-Rad) at 65 °C, using 5 mM H_2SO_4 as the mobile phase with a flow rate of 0.6 ml/min.

Quantification of *p*-CA was performed as described in Rodriguez et al. (2015) using a HPLC (Thermo Fisher Scientific), with a Discovery HS F5 150 mm × 2.1 mm column (particle size 3 μm). The samples were analyzed using a gradient method with two solvents: (A) 10 mM ammonium formate pH 3.0 and (B) acetonitrile at 1.5 ml min^1. The *p*-CA was detected by absorbance at 277 nm and the retention time was 4.7 min. The area under the curve was integrated with Chromeleon software 7. The quantification of *p*-CA was performed based on 5 points calibration curve in the range of 0.1–1 mM. For the dry cell weight measurement 5 ml of culture broth was filtered through a 0.45 μm filter membrane, after that the membrane was dried at 95 °C for 24 h and cooled down in a desiccator. The dry cell weight was calculated by measuring the weight increment of the dried filter.

2.4. Transcriptome analysis

Samples for RNA extraction were taken after four retention times of

Table 1
Plasmids used in this study.

Integrative plasmids			
Name	**Parent plasmid**	**Properties**	**Reference**
pCfB257		X-3, loxP, *KlLEU2*	Jensen et al. (2014)
pCfB258		X-4, loxP, *SpHIS5*	Jensen et al. (2014)
pCfB390		XI-3-loxP-*KlURA3*	Jensen et al. (2014)
pCfB0826	pCfB258	X-4, loxP, *SpHIS5*, BB0361(Sc*ARO7*^{G141S} < -), BB0010(< -P_{TEF1}-P_{PGK1}- >), BB0364(Sc*ARO4*^{K229L}- >)	Rodriguez et al. (2015)
pCfB03523	pCfB390	XI-3-loxP, *KlURA3*, BB0380(Fj_*TAL* < -), BB0008(P_{TEF1} < -),	This study
pCfB03524	pCfB390	XI-3-loxP, *KlURA3*, BB0380 (Fj_*TAL* < -), BB0010(< -P_{TEF1}-P_{PGK1}- >), BB0501(Ec*aroL*- >)	This study

steady-state fermentation by rapidly withdrawing 5 ml of culture and injecting it into a 50 ml falcon tube with ca. 30 ml of crushed ice, the samples were immediately centrifuged at 4000 rpm for 5 min at −20 °C. The supernatant was discarded, the pellet was frozen in liquid nitrogen and stored at −80 °C until further analysis. The RNA extraction was performed using the RNeasy Mini Kit (QIAGEN). The DNA was removed from the sample using Turbo DNA-free Kit (Ambion). The purified RNA samples were analyzed with a 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA) and stored at −80 °C until further analysis.

The sequencing libraries were prepared in four replicates using a TruSeq® Stranded mRNA sample preparation kit LT (Illumina Inc.). The final concentration of each cDNA library was measured by Qubit® 2.0 Fluorimeter and Qubit dsDNA Broad Range assay (Life Technologies). Average dsDNA library size was determined by using the Agilent DNA 1000 kit on an Agilent 2100 Bioanalyzer (Agilent Technologies). Libraries were normalized and pooled in 10 mM Tris-Cl, pH 8.0, plus 0.05% Tween 20 to the final concentration of 10 nM. Denaturated in 0.2 N NaOH, 1.2 pm pool of 16 libraries in 1300 µl ice-cold HT1 buffer was loaded into the flow cell provided in the NextSeq. 500/550 Mid Output v2 Reagent kit (150 cycles, Illumina Inc.). Libraries were sequenced on the NextSeq (Illumina Inc.) platform with a paired end protocol and read lengths of 75 nt. RNA-seq data have been deposited in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-6208.

2.5. Metabolomics analysis

Sampling, quenching and washing of the intracellular metabolites was performed as described by Canelas et al. (2009). The analysis of amino acids and other organic acids was performed according to Khoomrung et al. (2015). The analysis of the derivatized metabolites was performed using a Focus GC ISQ-LT single quadrupole GC-MS (Thermo Fisher Scientific, USA). The column was a Zebron ZB-1701 GC column (30 m 0.25 mm I.D., 0.25-mm film thickness, Phenomenex, Macclesfield, UK). The metabolites were identified by comparing their retention times and mass spectrum profiles with the authentic standards or the mass spectra from the National Institute of Standards and Technology (NIST), USA library. The data was processed using the Quan browser function in the Xcalibur software version 2.2 (Thermo Fisher Scientific).

2.6. Data analysis

The alignment of sequencing reads to the reference genome was performed using TopHat, the assembly and quantification of the expression levels was developed with Cufflinks and a preliminary analysis of the data was performed with CummeRbund, the methods were used as described by Trapnell et al. (2012). Paired comparisons were performed between optimized and non-optimized strains on each background: the strain ST4288 was compared with the strain ST4408 and the strain ST4397 was compared with the strain ST4353.

The gene set analysis was performed using the R package Piano (Väremo et al., 2013), a platform for integrative analysis of omics data. The p-values and the fold changes were used as input data and two types of analysis were performed with this program: first a gene-set analysis with the reporter algorithm for gene ontology (GO) and second a gene set analysis using the reporter metabolites. The gene-metabolite network was obtained from the *S. cerevisiae* metabolic model iTO977 (Österlund et al., 2013). The gene sets and reporter metabolites with a distinct directional p-value < 0.05 were chosen for the analysis.

The network topology analysis was performed using Kiwi a tool for visualization and interpretation of gene sets analysis (Väremo et al., 2014). This tool allows integrating the results of the gene set analysis with a gene set interaction network. The input for this analysis was a gene set interaction network obtained from the *S. cerevisiae* metabolic model iTO977 and the results from the gene set analysis using reporter metabolites.

For the metabolomics data, we did a PCA analysis in MATLAB to identify the differences between the four strains object of this research and to establish the differences between the engineered and non-engineered strain we did volcano plots based on the results of a *t*-test comparison between optimized and non-optimized strains on each background.

Table 2
Strains used in this study.

Strains				
Strain ID	**Parent strain**	**Genotype**	**Plasmids**	**Source**
ST10	–	CEN.PK102.5B MAT*a* *ura*3-52*his*3 Δ 1*leu*2-3/112 *MAL*2-8^{c} *SUC*2		Entian and Kötter (2007)
ST144	–	S288c [BY4741 MAT*a* *his*3Δ0 l*eu*2Δ0 *met*15Δ0 *ura*3Δ0]		Brachmann et al. (1998)
ST691	ST10	CEN.PK MAT*a* *aro10Δ* *pdc5Δ* *ura*3-52*his*3 Δ 1*leu*2-3/112 *MAL*2-8^{c} *SUC*2		Rodriguez et al. (2015)
ST4360	ST144	S288c MAT*a* *his*3Δ0 *leu*2Δ0 *ura*3Δ0		This study
ST4195	ST144	S288c MAT*a* *aro*10Δ *pdc*5Δ *ura*3Δ0 *his*3Δ0 *leu*2Δ0		This study
ST4408	ST10	P_{TEF1}- > Fj_*TAL*	pCfB257, pCfB258, p03523	This study
ST4288	ST691	P_{TEF1}- > Fj_*TAL*, P_{PGK1}- > Ec_*aroL*, P_{TEF1}- > Sc_*ARO7*^{G141s}, P_{PGK1}- > Sc_*ARO4*^{K229L}	pCfB257, pCfB826, p03524	This study
ST4353	ST4360	P_{TEF1}- > Fj_*TAL*, P_{PGK1}- > Ec_*aroL*, P_{TEF1}- > Sc_*ARO7*^{G141S}, P_{PGK1}- > Sc_*ARO4*^{K229L}	pCfB257, pCfB826, p03524	This study
ST4397	ST4195	P_{TEF1}- > Fj_*TAL*	pCfB257, pCfB258, p03523	This study

Table 3
Physiological data of the strains grown in batch and chemostat cultivations.

Background strain	**CEN.PK**		**S288c**	
Strain ID	ST4408	ST4288	ST4397	ST4353
Optimized for *p*-CA production	No	Yes	No	Yes
Batch				
Maximum specific growth rate μ_{max} (h^{-1})	0.334 ± 0.006	0.294 ± 0.006	0.292 ± 0.005	0.271 ± 0.009
Final titer of *p*-CA (g L^{-1})	0.202 ± 0.005	2.405 ± 0.054	0.081 ± 0.005	2.018 ± 0.000
Biomass yield on glucose (g g^{-1})	0.477 ± 0.044	0.268 ± 0.093	0.422 ± 0.005	0.352 ± 0.053
p-CA yield on glucose (g g^{-1})	0.001 + 0.000	0.013 + 0.000	0.000 ± 0.000	0.012 ± 0.000
Glycerol yield on glucose (g g^{-1})	0.019 ± 0.001	0.018 ± 0.002	0.048 ± 0.000	0.053 ± 0.001
Acetate yield on glucose (g g^{-1})	0.010 ± 0.003	0.014 ± 0.002	0.006 ± 0.002	0.011 ± 0.001
Ethanol yield on glucose (g g^{-1})	0.309 ± 0.001	0.293 ± 0.004	0.289 ± 0.013	0.297 ± 0.013
Final biomass dry weight (g DCW L^{-1})	13.198 ± 0.279	12.566 ± 0.056	11.481 ± 0.131	10.401 ± 0.785
Chemostat (steady-state data)				
Titer of *p*-CA (g L^{-1})	0.117 ± 0.000	0.507 ± 0.013	0.081 ± 0.005	0.410 ± 0.024
Biomass dry weight (g DCW L^{-1})	4.647 ± 0.156	4.670 ± 0.181	4.625 ± 0.165	4.620 ± 0.094
Glucose (g L^{-1})	ND	ND	ND	ND
Glycerol (g L^{-1})	ND	0.021 ± 0.004	ND	0.013 ± 0.001
Acetate (g L^{-1})	ND	ND	ND	ND
Ethanol (g L^{-1})	ND	ND	ND	ND

ND – not detected.

3. Results

3.1. Physiological characterization of low and high producers of p-coumaric acid

To understand the fundamental metabolic changes triggered by the overproduction of *p*-CA, and the response of different background strains to these changes, we constructed two strains in each of the genetic backgrounds (CEN.PK and S288c). The “low-producers” were generated by overexpressing tyrosine ammonia lyase from *Flavobacterium johnsoniae* under control of the P_{TEF1} promoter. The “high-producers” were created by additional overexpression of *aroL* from *Escherichia coli* under control of the P_{TEF1} promoter, *ARO7*^{G141S} and *ARO4*^{K229L} from *S. cerevisiae* under control of the promoters P_{TEF1} and P_{PGK1} respectively and deletion of *ARO10* and *PDC5*. The resulting 4 strains were analyzed in batch and glucose-limited chemostat cultivations.

The concentrations of *p*-CA in batch and continuous cultivations were higher in the CEN.PK strains in comparison to the S288c strains with the same genetic modifications (Table 3). The glycerol yield was higher in the S288c strains in comparison to the CEN.PK strains. In batch fermentations, the optimized strains had lower biomass yield and accumulated more acetate than the non-optimized ones.

3.2. Transcriptional response of the strains to the synthesis of p-coumaric acid

For analysis of the differential gene expression, we did pairwise comparisons between the optimized and non-optimized strains for *p*-CA production: CEN.PK strains (ST4288 and ST4408) and the S288c strains (ST4353 and ST4397).

Significantly up and down-regulated gene sets were identified through a gene set analysis using GO terms (p adjusted value < 0.05). The engineered high-producing CEN.PK strain did not have any gene sets that were significantly upregulated in comparison to the low-producing CEN.PK strain. For S288c strain, however, gene sets related to DNA helicase activity, telomere maintenance and ribonuclease activity were upregulated (Fig. 1). Among the down-regulated gene sets were transport functions and iron metabolism, which was observed for both strain backgrounds. Additionally, S288c strain had remarkable down-regulations in gene sets related to the synthesis of amino acids and proteins (Fig. 1).

To elucidate the biological connections between the gene sets, we did a network analysis of the gene sets using metabolite reporters (Patil and Nielsen, 2005; Väremo et al., 2013), and used the network visualization tool Kiwi for visualizing the results (Väremo et al., 2014). The network analysis allows integrating the information from the gene set analysis with metabolite interactions from a genome-scale metabolic model of yeast provided via the PIANO toolbox (Väremo et al., 2013).

For the CEN.PK strain we observed significant downregulation of genes correlated to eight amino acids, proton H(+) and galactose. All the amino acids mapped in the network have in common upregulations in *AGP1* and *GAP1* together with downregulations in *BAP3* and *BAP2* and *TAT1*, all of them are involved in the transport of amino acids (Fig. 2A, Fig. S1).

One of the metabolites correlated to downregulated genes is L-tyrosine, the precursor of *p*-CA, the network analysis shows that besides correlation to downregulation of transporters, this metabolite is related to a strong upregulation in the aromatic aminotransferase II *ARO9* involved in the conversion of *p*-hydroxyphenylpyruvate into L-tyrosine, i.e. the first step of tyrosine catabolism. Finally, proton H(+) is mainly correlated to downregulations in genes involved in transport functions (*BAP2*, *TAT1*, *ALP1*, *TPO1*, *BIO5*, *VHT1*) and D-galactose is mainly correlated to downregulation of hexose transport (*HXT10*, *HXT14*).

The strain S288c had downregulated genes correlated to 5 amino acids (L-methionine, L-tyrosine, L-tryptophan, L-glutamate and L-ornithine), three sugars (D-fructose, alpha-D-glucose and alpha-D-mannose), acetaldehyde and 2-oxoglutarate (Fig. 2B, Fig. S2).

The amino acids reported in the network analysis had in common the downregulation of *BAP2*, *TAT1* and the upregulation of *AGP1*. These genes are correlated to amino acid transmembrane transporter activity. We found two aromatic amino acids in the network L-tyrosine and L-tryptophan, they share downregulations in genes related to amino acids transport (*BAP2* and *TAT1*), and they differ in the upregulations when L-tyrosine has a strong upregulation in *ARO9* and *ALD3*. L-tryptophan has a strong upregulation in *MSY1* (Fig. 2B, Fig. S2).

Finally, the metabolites 2-oxoglutarate, L-glutamate and L-ornithine are correlated to downregulated genes involved in the synthesis and transport of amino acids. L-glutamate has a central role in the metabolic network and it is correlated to downregulation of *ASN1*, *ADE4*, *CAR2* and *TRP2*, genes involved in the synthesis of amino acids. The three sugars reported in the network are correlated to downregulations in genes involved in the transport of sugars (*HXT2* and *HXT16*).

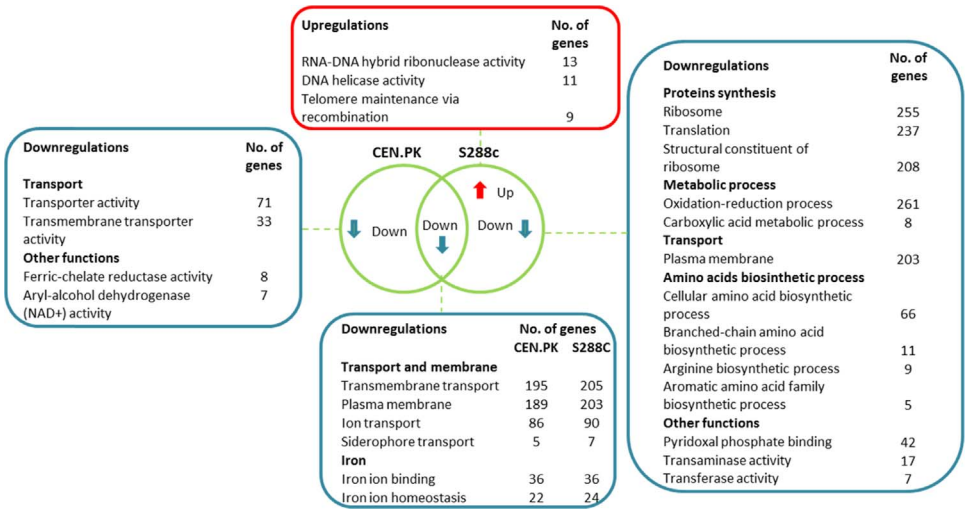

Fig. 1. Gene sets with significant differences in the optimized strains for *p*-CA production in comparison to the non-optimized strains.

3.3. Changes of intracellular metabolome in response to the synthesis of p-coumaric acid

To identify the differences in the metabolome caused by over-production of *p*-CA and by different genetic backgrounds, we did a PCA analysis. The first component of the PCA accounted for 82% of the variability and showed significant differences between the background strains CEN.PK and S288c, the metabolites with higher contributions to this component are phosphoric acid, disilaheptane, L-ornithine, glutamic acid, lysine and citrate, all of them related to higher concentration of the metabolites in the CEN.PK background whereas the strain S288c is related to higher values of *cis*-9-hexadecanoic acid (Fig. 3).

The second component explains 8% of the variability and establishes the differences between optimized and non-optimized strains for the two backgrounds tested, the metabolites with higher contributions to this component are glutamine and L-tyrosine with higher concentrations for the engineered strains whereas the non-optimized strains are related to higher concentrations of malic acid.

A *t*-test was performed comparing the non-optimized and optimized strain of each background, aiming to identify which metabolites has significant differences when the cells are producing *p*-CA. The significant differences for both of the background strains tested are mainly related to the low concentration of metabolites in the optimized strains. For the CEN.PK strains, we found significant differences in four metabolites: two amino acids (L-valine and L-threonine), malic and citric

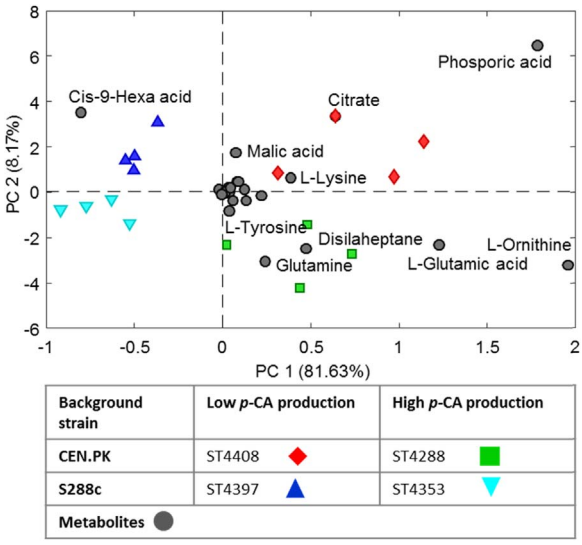

Fig. 3. Score and loading plot from the principal component analysis based on the metabolome data of optimized and non-optimized strains in the *S. cerevisiae* backgrounds CEN.PK and S288c.

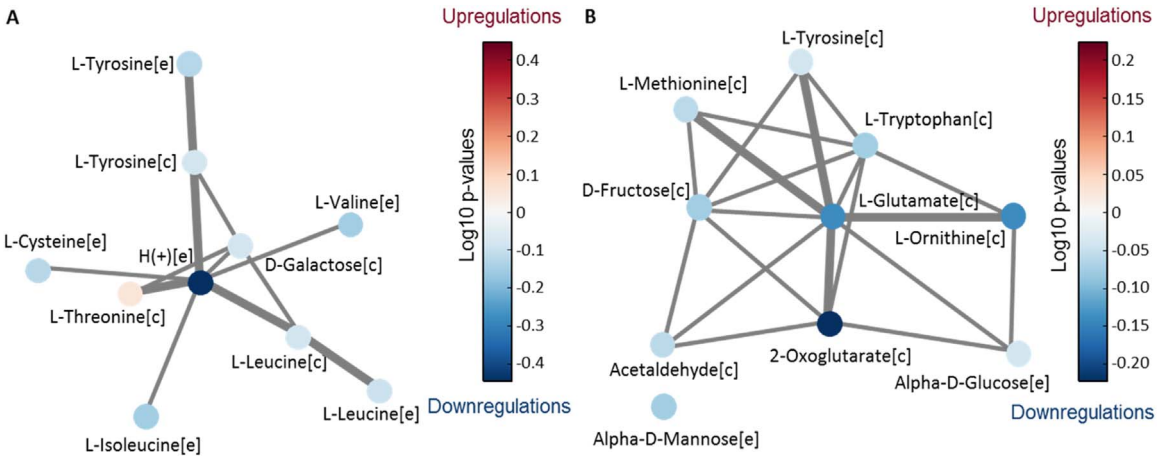

Fig. 2. Network topology analysis identified metabolites with significant differences in the optimized strains in comparison to the non-optimized strains. The nodes are resized according to the gene-set significance, the colors reflect the direction of change of the gene-set, the edges between two metabolites symbolized how close they are in the metabolite-metabolite network and the thickest edges link the metabolites that are in close proximity to each other. A. comparison between CEN.PK strains; B. Comparison between S288c strains.

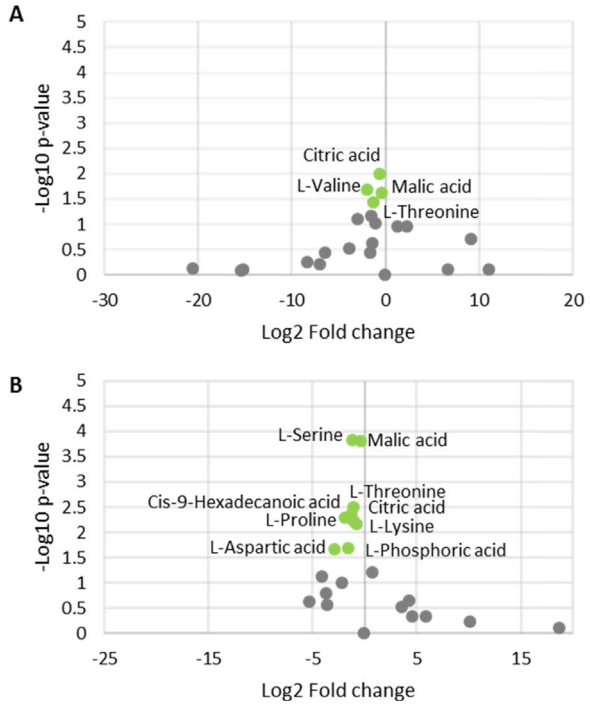

Fig. 4. Volcano plot based on the statistical significance of the *t*-test and fold change from the comparison of the reporter metabolites between optimized and non-optimized strain on each background. A. CEN.PK strains, B. S288c strains.

acid. For the S288c strain, we found significant differences in 5 amino acids, phosphoric acid, malic acid, citric acid, and *cis*-9-hexadecanoic acid (Fig. 4). There is bigger variation between the low and high-producing S288c strains than CEN.PK strains.

3.4. Engineering of highly upregulated or downregulated genes

As the next step, we wanted to investigate how *p*-coumaric acid production could be influenced by targeted deletion or upregulation of the genes that showed significant changes in the transcriptome analysis. We chose 24 genes that were significantly upregulated or downregulated. We carried out single knock-outs or overexpressions of these genes in strains ST4964 and ST4965, which were generated from correspondingly strains ST4288 and ST4408 by additional expression of a *cas9* gene to enable simplified genome editing (Stovicek et al., 2015). Knock-outs of 9 genes could not be obtained. It may either be due to inefficient guiding gRNA or due to conditional lethality of these genes. The overexpression was realized by inserting an extra copy of the gene under control of a strong constitutive promoter *TEF1*. All the resulting strains were cultivated in feed-in-time medium and the optical densities and titer of *p*-coumaric acid were measured. None of the overexpressions resulted in significant change in the *p*-CA titer in either a low-producing nor high-producing strains (Fig. S3). The deletions did not influence *p*-CA titer in the low-producing strains, however, we observed improvement of *p*-CA production for seven gene deletions in the high-producer strain ST4964 background (Fig. S4, Table 4). All the seven deletion targets that triggered higher amounts of *p*-CA were involved in the transport of amino acids, polyamines, and sugar.

The highest improvement in *p*-CA synthesis was obtained by deleting *TAT1,* encoding a tyrosine and tryptophan amino acid transporter. The knockout of this gene triggered a 50% increase of *p*-CA titer. Strains with knockouts of polyamine transporter *TPO1* and arginine transporter *ALP1* resulted in 40–45% higher titer. Finally, deletion of amino acids transporters (*BAP2*, *AGP3*), acetate transporter *ADY2* and galactose transporter *GAL2* gave 20–30% improvement (Table 4, Figs. S4 and S5).

Table 4
Percentage of *p*-CA titer improvement resulting from deletion of downregulated genes.

Strain	Name	Name description	% improvement of *p*-CA titer
ST5935	*TAT1*	Tyrosine and tryptophan amino acid transporter	48 ± 21
ST5937	*TPO1*	Transporter of polyamines	45 ± 13
ST5938	*ALP1*	Arginine transporter	39 ± 11
ST5939	*AGP3*	High-affinity glutamine permease	27 ± 29
ST5940	*ADY2*	Acetate transporter	26 ± 16
ST5942	*GAL2*	Galactose permease	23 ± 10
ST5949	*BAP2*	Branched-chain amino acid permease	22 ± 25

4. Discussion

In our study, engineered high-producer strain with CEN.PK background gave 20–25% higher *p*-CA titers in batch and continuous cultivations in comparison with S288c strain engineered identically. Moreover, the CEN.PK-producer had a higher maximum specific growth rate μ_{max} than S288c-producer. Transcriptome analysis showed that the CEN.PK strain was less affected by engineering towards higher *p*-CA production than the S288c strain, as the number of significantly up-/down-regulated genes was correspondingly 652 and 1927 amongst others, strain S288c had downregulations in gene sets involved in amino acid and protein biosynthesis. This suggests that CEN.PK may be a better platform strain for production of aromatic compounds than the S288c strain.

The transcriptome analysis also revealed downregulations in transport functions in the engineered strains of both backgrounds, which could be a response to the stress triggered by production of *p*-CA. Previous studies on plasma membrane integrity and ethanol stress in *S. cerevisiae* have reported that yeast cells react to chemical stress by downregulating the transport of some metabolites and by decreasing gene expression in energy-demanding processes (Stanley et al., 2010, Madeira et al., 2010; Leao and van Uden, 1984).

For the engineered S288c strain, metabolomics analysis revealed lower concentrations of phosphoric acid, L-ornithine and glutamic acid, which correlates with downregulation of the gene sets involved in the synthesis of proteins and amino acids. Interestingly previous research on *S. cerevisiae* stress responses to oxidative stress had shown diminution in the synthesis of proteins as a prevention mechanism under potentially error-prone conditions (Shenton and Grant, 2003a).

We observed higher accumulation of glycerol by the engineered strains than in non-optimized strains. Synthesis of glycerol is known as an important factor in the control of osmoregulation and of redox balance (Hohmann et al., 2007; Muzzey et al., 2009). Another interesting metabolite is *cis*-9-hexadecanoic acid; this metabolite had higher concentrations in the S288c strain in comparison to CEN.PK strain; we propose that the stress originating from the production of *p*-CA may trigger the synthesis of this fatty acid in the S288c strains. It had been previously reported that genetically engineered strains with a higher concentration of *cis*-9-hexadecanoic acid were more tolerant to temperature and oxidative stress (Steels et al., 1994; Jamieson, 1998).

Overexpression or knock-outs of genes with changed transcriptional profile did not affect *p*-CA production in low-producing strain background (ST4965). Most likely, in this strain the flux is primarily limited by the biosynthesis of tyrosine precursor and hence modulating the expression of primarily transport and stress-related genes could not influence the production significantly. The situation was different for the high-producing strain background (ST4964), where knock-outs of seven transport-related genes improved *p*-CA titer by 20–50%. Particularly, the deletion of the tyrosine and tryptophan transporter *TAT1* resulted in 50% improvement of *p*-CA titer. Possibly, the deletion of *TAT1* resulted in reduction of the leakage of tyrosine from the cells,

hence more could serve as substrate for *p*-CA production. Interestingly, overexpression of the same genetic targets did not lead to any significant changes in *p*-CA titer. The number of transporter proteins per cell is one-two orders of magnitude lower than the cytosolic proteins and overexpression of a transporter gene may not result in a significant increase of transporter protein content simply due to the membrane crowding.

Our study highlights the importance of transporters when engineering cell factories for production of small molecules. Future studies are warranted on identifying the transporters responsible for the efflux of *p*-CA and intermediates and on using this knowledge for further strain improvement.

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Declaration of interest

The authors declare no competing interests.

Contributions

AR, IB and JN conceived and designed the study and analyzed the results. AR, YC and SK did the experimental work. AR, YC, EO and SK processed and analyzed the data. AR and IB drafted the manuscript and all authors read, edited and approved the final manuscript.

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.2017.10.013.

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