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Special Issue Article

Deletion of the *Saccharomyces cerevisiae ARO8* gene, encoding an aromatic amino acid transaminase, enhances phenylethanol production from glucose

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Abstract

Phenylethanol has a characteristic rose-like aroma that makes it a popular ingredient in foods, beverages and cosmetics. Microbial production of phenylethanol currently relies on whole-cell bioconversion of phenylalanine with yeasts that harbour an Ehrlich pathway for phenylalanine catabolism. Complete biosynthesis of phenylethanol from a cheap carbon source, such as glucose, provides an economically attractive alternative for phenylalanine bioconversion. In this study, synthetic genetic array (SGA) screening was applied to identify genes involved in regulation of phenylethanol synthesis in *Saccharomyces cerevisiae*. The screen focused on transcriptional regulation of *ARO10*, which encodes the major decarboxylase involved in conversion of phenylpyruvate to phenylethanol. A deletion in *ARO8*, which encodes an aromatic amino acid transaminase, was found to underlie the transcriptional upregulation of *ARO10* during growth, with ammonium sulphate as the sole nitrogen source. Physiological characterization revealed that the *aro8Δ* mutation led to substantial changes in the absolute and relative intracellular concentrations of amino acids. Moreover, deletion of *ARO8* led to *de novo* production of phenylethanol during growth on a glucose synthetic medium with ammonium as the sole nitrogen source. The *aro8Δ* mutation also stimulated phenylethanol production when combined with other, previously documented, mutations that deregulate aromatic amino acid biosynthesis in *S. cerevisiae*. The resulting engineered *S. cerevisiae* strain produced >3 mM phenylethanol from glucose during growth on a simple synthetic medium. The strong impact of a transaminase deletion on intracellular amino acid concentrations opens new possibilities for yeast-based production of amino acid-derived products. **Copyright © 2014 John Wiley & Sons, Ltd.**

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Introduction

The characteristic rose-like aroma of phenylethanol makes it one of the most-used chemicals in the cosmetics industry (Fabre *et al.*, 1998). Moreover, phenylethanol is used as an additive in foods and beverages. Most phenylethanol is currently synthesized chemically via a Friedel–Craft reaction that leads to the accumulation of undesired by-products and increases purification costs (Etschmann *et al.*, 2002). Chemical production of phenylethanol restricts its applications, since aroma compounds

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should be naturally produced, especially in foods and beverages. To label a product as 'natural', it has to derive from natural sources via a physical, enzymatic or microbiological process (US Food and Drug Administration, http://www.fda.gov/Food/GuidanceRegulation/). Extraction of phenylethanol from plants and flowers leads to low product recovery and, consequently, high costs (Eikani *et al.*, 2005). A much more effective production method is whole-cell bioconversion of phenylalanine (Eshkol *et al.*, 2009; Etschmann *et al.*, 2005; Stark *et al.*, 2003).

Many microorganisms, and in particular several yeasts, produce phenylethanol from phenylalanine via a pathway first described over a century ago (Ehrlich and Herter, 1904; see also Hazelwood *et al.*, 2008). In *Saccharomyces cerevisiae* import of phenylalanine is mediated by either the general amino acid transporter Gap1 or the low-affinity amino acid permease Agp1 (Forsberg *et al.*, 2001). Once inside the cell, phenylalanine is then transaminated to phenylpyruvate by either of two differentially expressed aromatic amino acid transaminases, Aro8 and Aro9. *ARO8* is constitutively expressed and, under many conditions, involved in aromatic amino acid biosynthesis. Conversely, transcription of *ARO9* is induced by aromatic amino acids, suggesting a key role in their catabolism via the Ehrlich pathway (Iraqui *et al.*, 1998; Kradolfer *et al.*, 1982). Aro8 and Aro9 exhibit different kinetic properties, but null mutations in either of the two genes can be partially complemented by the other (Urrestarazu *et al.*, 1998). Aro8 has a broad substrate specificity, accepting all three aromatic amino acids as well as leucine, methionine and glutamate as amino donors with the corresponding 2-oxo-acids as amino acceptors (Urrestarazu *et al.*, 1998). Aro8 has recently also been implicated in lysine biosynthesis, where it transfers the amino group from glutamate to 2-oxoadipate to form 2-oxoglutarate and 2-aminoadipate (Bulfer *et al.*, 2013). In contrast, Aro9 cannot use 2-oxoglutarate as amino acceptor with tryptophan, leucine or methionine as amino donors (Urrestarazu *et al.*, 1998).

Decarboxylation of the 2-oxo acid generated upon amino acid transamination is the only irreversible step in the Ehrlich pathway. In *S. cerevisiae*, decarboxylation of phenylpyruvate to phenylacetaldehyde is primarily catalysed by the broad-substrate thiamine pyrophosphate (TPP)-dependent 2-oxo acid decarboxylase Aro10 (Vuralhan *et al.*, 2003). The pyruvate–decarboxylase isoenzyme Pdc5 can also catalyse this reaction, but its kinetic parameters for phenylpyruvate decarboxylation are inferior to those of Aro10 (Romagnoli *et al.*, 2012). The metabolic fate of phenylacetaldehyde formed in the decarboxylation reaction depends on growth conditions. In aerobic glucose-grown batch cultures, with phenylalanine as sole nitrogen source, *S. cerevisiae* produces a mixture of phenylethanol and phenylacetate in a 9:1 ratio. Growth under anaerobic conditions results in accumulation of phenylethanol (Vuralhan *et al.*, 2003, 2005). The identity of the oxidoreductase responsible for the last step of the Ehrlich pathway is not clear, but it has been shown that any of the ethanol dehydrogenases (Adh1, Adh2, Adh3, Adh4 and Adh5) or the formaldehyde dehydrogenase Sfa1 can convert phenylacetaldehyde into phenylethanol (Dickinson *et al.*, 2003).

Transcription of the structural genes encoding the Ehrlich pathway enzymes and, presumably, their *in vivo* activity, is regulated in two complementary ways. In the presence of an aromatic amino acid, transcriptional activation is mediated by Aro80 (Iraqui *et al.*, 1999; Lee and Hahn, 2013; Vuralhan *et al.*, 2003, 2005), while nitrogen catabolite repression (NCR) occurs when a preferred nitrogen source (i.e. ammonium or glutamine) is available (Boer *et al.*, 2007). Conditions for efficient induction are therefore met during bioconversion of phenylalanine, when phenylalanine is present and ammonium absent. However, phenylalanine is not a cheap precursor and *de novo* production of phenylethanol from glucose would be economically attractive. In a previous study, elimination of the feedback inhibition of phenylalanine and tyrosine on DAHP synthase (Aro3 and Aro4) and of the chorismate mutase (Aro7) (Krappmann *et al.*, 2000) yielded *S. cerevisiae* strains able to produce phenylethanol during growth on glucose with ammonium sulphate as the nitrogen source (Luttik *et al.*, 2008). However, the impact of transcriptional deregulation of the Ehrlich pathway genes has not yet been explored.

The goal of the present study was to identify genes that influence the transcriptional (de)repression of the Ehrlich pathway during growth with ammonium as the nitrogen source. With the aid of synthetic genetic array (SGA) technology, we constructed a strain collection in which deletions of the non-essential genes in the *S. cerevisiae* genome were combined with a reporter plasmid, comprising the *ARO10* promoter fused to a reporter

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gene (*yEGFP*) encoding a fluorescent reporter protein. After screening by flow cytometry, it was found that deletion of *ARO8* led to a deregulated expression of the *ARO10* promoter. The impact of this deletion was further studied by transcriptome and intracellular metabolite analyses. Finally, phenylethanol production was compared with strains that combined the *aro8Δ* mutation with mutations that were previously shown to deregulate aromatic amino acid biosynthesis (Krappmann *et al.*, 2000; Luttik *et al.*, 2008).

Materials and methods

Yeast strains and maintenance

The *Saccharomyces cerevisiae* strains used in this study (Table 1) were constructed in the CEN.PK and BY4741 backgrounds (Brachmann *et al.*, 1998; Entian and Kötter, 2007; Nijkamp *et al.*, 2012). Yeast strains were maintained on YPD medium (10 g/l yeast extract (BD Difco, Breda, The Netherlands); 20 g/l peptone (BD Difco); 20 g/l glucose in demineralized water). Culture stocks were prepared from shake-flask cultures incubated at 30°C and stirred at 200 rpm, by addition of 30% v/v glycerol and were stored at −80°C.

Media and culture conditions

Shake-flask growth experiments were conducted in synthetic medium (SM) containing 5 g $(NH_4)_2SO_4$, 3 g KH_2PO_4, 0.5 g $MgSO_47H_2O$, 20 g glucose, 1 ml trace element solution, 1 ml vitamin solution and 8% antifoam-C emulsion (Sigma-Aldrich, Zwijndrecht, The Netherlands) per litre of demineralized water. Trace elements and vitamin solutions were prepared and sterilized as described previously (Verduyn *et al.*, 1992). Glucose was added to a final concentration of 20 g/l. SM for growth experiments with amino acid as sole nitrogen source was prepared by replacing $(NH_4)_2SO_4$ by either 5.0 g/l L-phenylalanine or 10.0 g/l L-leucine. These media were also supplemented with 3.3 g/l K_2SO_4 to compensate for the lack of ammonium sulphate. If required, 0.15 g/l uracil, 0.2 g/l G418, 0.2 g/l hygromycin or 1.8 g/l acetamide were added to the medium. 500 ml or 250 ml flasks containing 100 ml or 20 ml liquid medium, respectively, were incubated in an orbital shaker (New Brunswick Scientific, Edison, NJ, USA) at 200 rpm and at 30°C. Agar plates were made by adding 20 g/l agar to liquid media. Plates used for selection of yeast strains transformed with the amdSYM marker were prepared as described previously (Solis-Escalante *et al.*, 2013). The medium composition for the SGA experiments is described in Table S1 (see supporting information).

Chemostat cultivation

S. cerevisiae strains were grown in aerobic glucose-limited chemostat cultures on a synthetic medium (Boer *et al.*, 2005, 2007). Chemostat cultivations were performed in 2 litre bioreactors (Applikon, Schiedam, The Netherlands), with a working volume of 1 litre and a dilution rate of 0.10 h^{-1}, as described previously (Tai *et al.*, 2005). Chemostat cultures were assumed to be in steady state when, at least five volume changes after initiating continuous feeding, culture dry weight and off-gas CO_2 analyses differed by $< 2\%$ over two consecutive volume changes.

Strain construction

Oligonucleotide primers and plasmids used in this study are shown in Tables 2 and 3, respectively. Gene deletions were performed using the loxP–marker–loxP recombinase system, using pUG6 (Güldener *et al.*, 1996), pUGamdSY (Solis-Escalante *et al.*, 2013) and pUGHphNT1 (de Kok *et al.*, 2012) as templates for amplification of knock-out cassettes. The primers included 50 bp tails homologous to the promoter and the terminator (primers 'del Fw' and 'del Rv', respectively) of the targeted gene. Deletion cassettes were transformed into *S. cerevisiae* by the lithium acetate method (Gietz and Woods, 2002). Transformants were selected on plates containing G418, hygromycin or acetamide. Correct integration of deletion cassettes was verified via colony PCR with DreamTaq DNA polymerase (Fisher scientific, Landsmeer, The Netherlands) with a forward primer, 'F', that annealed upstream of the insertion point and two reverse primers: primer 'E' annealing in the gene open reading frame as negative control and primers KanA, RvAmdS and HphN Rv that annealed inside the deletion cassette.

For construction of an *aro8Δ aro80Δ* double mutant, the *ARO8* gene was deleted in strain IMK431 (*aro80Δ*::*kanMX*). To prevent recombination of the homologous regions present in the deletion cassettes, a special cassette with long

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Table 1. *S. cerevisiae* strains used in this study

Strains	Characteristic	Reference
CEN.PK113-7D	*MAT***a**	(Nijkamp *et al.*, 2012)
CEN.PK113-5D	*MAT***a** *ura3-52*	(Entian and Kötter, 2007)
IME185	*MAT***a** *ura3-52* pUDC071 [2μ *URA3 ARO10*$_{pr}$*-yEGFP-CYC*$_{ter}$]	This study
BY4741	*MAT***a** *his3Δ1 leu2Δ met15Δ ura3Δ*	EUROSCARF
BY4742	*MATα his3Δ1 leu2Δ lys2Δ ura3Δ*	EUROSCARF
IMX100	*MATα his3Δ1 leu2Δ lys2Δ ura3Δ* pXP346 [*can1Δ::LEU2-MFA1*$_{pr}$*-HIS3*]	This study
IMX101	*MATα his3Δ1 leu2Δ lys2Δ ura3Δ* pXP346 [*can1Δ::LEU2-MFA1*$_{pr}$*-HIS3*] pUDC071 [2μ *URA3 ARO10*$_{pr}$*-yEGFP-CYC*$_{ter}$]	This study
IMC063	BY4741 *ura3Δ leu2Δ his3Δ met15Δ* pUDCTAR	This study
IMY065	IMC063 *aro8Δ::loxP-kanMX-loxP*	This study
IMY067	IMC063 *aro80Δ::loxP-kanMX-loxP*	This study
IMY070	IMC063 *npr2Δ::loxP-kanMX-loxP*	This study
IMY072	IMC063 *aro8Δ::loxP-kanMX-loxP* pUDC071 [2μ *URA3 ARO10*$_{pr}$*-yEGFP-CYC*$_{ter}$]	This study
IMY074	IMC063 *aro80Δ::loxP-kanMX-loxP* pUDC071[2μ *URA3 ARO10*$_{pr}$*-yEGFP-CYC*$_{ter}$]	This study
IMY078	IMC063 *npr2Δ::loxP-kanMX-loxP* pUDC071[2μ *URA3 ARO10*$_{pr}$*-yEGFP-CYC*$_{ter}$]	This study
IMY079	IMC063 pUDC071 [2μ *URA3 ARO10*$_{pr}$*-yEGFP-CYC*$_{ter}$]	This study
IMY092	IMC063 *rmd11Δ::loxP-kanMX-loxP*	This study
IMY093	IMC063 *rmd11Δ::loxP-kanMX-loxP* pUDC071[2μ *URA3 ARO10*$_{pr}$*-yEGFP-CYC*$_{ter}$]	This study
IMK525	*MAT***a** *ura3-52 aro8Δ::loxP-kanMX-loxP*	This study
IMK552	*MAT***a** *ura3-52 aro8Δ::loxP-amdSYM-loxP*	This study
IMK431	*MAT***a** *ura3-52 aro80Δ::loxP-kanMX-loxP*	This study
IMK497	*MAT***a** *ura3-52 npr2Δ::loxP-amdSYM-loxP*	This study
IMK498	*MAT***a** *ura3-52 rmd11Δ::loxP-amdSYM-loxP*	This study
IMZ461	*MAT***a** *ura3-52 aro80Δ::loxP-kanMX-loxP* pUDC071[2μ *URA3 ARO10*$_{pr}$*-yEGFP-CYC*$_{ter}$]	This study
IMZ437	*MAT***a** *ura3-52 aro8Δ::loxP-kanMX-loxP* pUDC071[2μ *URA3 ARO10*$_{pr}$*-yEGFP-CYC*$_{ter}$]	This study
IMZ430	*MAT***a** *ura3-52 npr2Δ::loxP-amdSYM-loxP* pUDC071[2μ *URA3 ARO10*$_{pr}$*-yEGFP-CYC*$_{ter}$]	This study
IMZ432	*MAT***a** *ura3-52 rmd11Δ::loxP-amdSYM-loxP* pUDC071[2μ *URA3 ARO10*$_{pr}$*-yEGFP-CYC*$_{ter}$]	This study
IMY081	*MAT***a** *ura3-52 aro8Δ::loxP-kanMX-loxP* pRS316	This study
IMK559	*MAT***a** *ura-52Δ aro80Δ::loxP-kanMX-loxP aro8Δ::loxP-amdSYM-loxP*	This study
IMZ458	*MAT***a** *ura3-52 aro80Δ::loxP-kanMX-loxP aro8Δ::loxP-amdSYM-loxP* pUDC071[2μ *URA3 ARO10*$_{pr}$*-yEGFP-CYC*$_{ter}$]	This study
IMN002	*MAT***a** *ura3-52 aro3Δ::loxP-kanMX-loxP TDH3*$_{pr}$*-ARO4*^{K229L}	(Luttik *et al.*, 2008)
IMN004	*MAT***a** *ura3-52 aro3Δ::loxP-kanMX-loxP TDH3*$_{pr}$*-ARO4*^{K229L} pUDE004 [2μ *URA3 TDH3*$_{pr}$*-ARO7*^{G141S}*-CYC*$_{ter}$]	(Luttik *et al.*, 2008)
IMN013	*MAT***a** *ura3-52 aro3Δ::loxP-kanMX-loxP TDH3*$_{pr}$*-ARO4*^{K229L} *aro8Δ::loxP-amdSYM-loxP*	This study
IMN014	*MAT***a** *ura3-52 aro3Δ::loxP-kanMX-loxP TDH3*$_{pr}$*-ARO4*^{K229L} *aro8Δ::loxP-amdSYM-loxP* pUDE004 [2μ *URA3 TDH3*$_{pr}$*-ARO7*^{G141S}*-CYC*$_{ter}$]	This study
IMK565	*MAT***a** *ura3-52 aro8Δ::loxP-kanMX-loxP tyr1Δ::loxP-HphN-loxP*	This study
IMK566	*MAT***a** *ura3-52 aro3Δ::loxP-kanMX-loxP TDH3*$_{pr}$*-ARO4*^{K229L} *aro8Δ::loxP-amdSYM-loxP tyr1Δ::loxP-HphN-loxP*	This study
IMN016	*MAT***a** *ura3-52 aro3Δ::loxP-kanMX-loxP TDH3*$_{pr}$*-ARO4*^{K229L} *aro8Δ::loxP-amdSYM-loxP tyr1Δ::loxP-HphN-loxP* pUDE004[2μ *URA3 TDH3*$_{pr}$*-ARO7*^{G141S}*-CYC*$_{ter}$]	This study

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Table 2. Oligonucleotide primers used in this study

Primer name	Sequence 5′→3′
Deletion primers	
ARO8 del forward	AACCCTGCAGTTGATACAGACATTGAATAGGACAACCGATCGTTACTATCGCCAGCTGAAGCTTCGTACGC
ARO8 del reverse	CGTACGTCCTTTTTTCACCTTATATATATTCTTCCAACGTATTTACCTCTGCATAGGCCACTAGTGGATCTG
NPR2 del forward	CCCTCTATCACTTGTTGCCTGTATCTCCGAATAGGACTGATAAGTGATAACAGCTGAAGCTTCGTACGC
NPR2 del reverse	ACGTTTGGATTCGTGTGTACTATCTTTATTTACGGAAATAAGTTGTAATAGCATAGGCCACTAGTGGATCTG
RMD11 del forward	ATCTTGAAATACCATTAAACATAGATAAGCTATTGAAATGTGAAGAAAATCAGCTGAAGCTTCGTACGC
RMD11 del reverse	TTCTTCCTTTTGTCTATTCTTATATACACATATTTTAATTTTTGGTAGATGCATAGGCCACTAGTGGATCTG
ARO80 del forward	GATCCACGCATAATAAGGTTACATTAAGCACTGCTTTATCTTTTATGTCTGCCAGCTGAAGCTTCGTACGC
ARO80 del reverse	GGTTGTCTTGGTTGATGACGTAATTCTTTGATATCTACTTATTTACGCGTTATTGGCCGCATAGGCCACTAGTGGATCTG
Control primers	
ARO8 Big del reverse	AGTCAAAGTCACGCCGCTTC
ARO80 F	TCGAGGAGCTGGATGCTTTAG
ARO80 E	CCGGACCAAGATCACATTTCAC
NPR2 F	TATGACTCACCCGGAAACCAC
NPR2 E	ATAGCTGGTTCGTAGGGAGAC
RMD11 F	TGCCATCTCCTAGAATCGAACC
RMD11 E	AGTAGCCGCAGGAATTGAAC
ARO80 F	TCGAGGAGCTGGATGCTTTAG
ARO80 E	CCGGACCAAGATCACATTTCAC
HphN reverse	GCATAGGCCACTAGTGGATCTG
Amds	TTCACCAGCAGCCAACTTAG
KanA	CGCACGTCAAGACTGTCAAG
LEU2A	ACTATATGTGAAGGCATGGCTATGGCACGGCAGACATTCCGCCAGATCATCAATAGGCACGTTTGGCCGAGCGGTCTAAG
LEU2F	TGCCGAACTTTCCCTGTATGAAGCGATCTGACCAATCCTTTGCCGTAGTTTCAACGTATGTGGGAAATGCTTCAAGAAGGTATTG
MET15G	GCCAGAGGTATAGACATAGCCAGACCTACCTAATTGGTGCATCAGGTGGTCATGGCCCTTTATCACGAGGCCCTTTCGTC
MET1H	GTTGAACATTCTTAGGCTGGTCGAATCATTTAGACACGGGCATCGTCCTCTCGAAAGGTGCGTTGGAGTCCACGTTCTTT
HIS3B	CACCTTTCGAGAGGACGATGCCCGTGTCTAAATGATTCGACCAGCCTAAGAATGTTCAACGGCTTAACTATGCGGCATCAG
HIS3I	GCCTACGGTTCCCGAAGTATGCTGCTGATGTCTGGCTATACCTATCCGTCTACGTGAATATTGCCGATTTCGGCCTATTG
Others	
ARO10 pr FW *Sac*I	CGGGAGCTCCTCTTGGTATTGCGTCTCC
ARO10 pr RV *Hin*dIII	GGGCAAGCTTGCTTAAGGGAGTTTCTTTGTTATC
Aro10 KO CHK	TGCTTGTACACCTCATGTAG
FK098	AACTTGTGGCCGTTTACGTC

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Table 3. Plasmids used in this study

Plasmid	Characteristics	Reference
pUG6	PCR template for *loxP-KanMX4-loxP* cassette	(Güldener *et al.*, 1996)
pUGamdSY	PCR template for *loxP-amdSYM-loxP* cassette	(Solis-Escalante *et al.*, 2013)
pUGHpHNT1	PCR template for *loxP-hphNT1-loxP* cassette	(de Kok *et al.*, 2012)
pAG416GAL–ccdB*yEGFP*	2μ ori *URA3* GAL_{pr}-*ccdB-yEGFP-CYC1*$_{ter}$	(Alberti *et al.*, 2007)
pRS316	*CEN6-ARS4 URA3*	(Sikorski and Hieter, 1989)
pRS411	*CEN6-ARS4* ori *MET15*	(Sikorski and Hieter, 1989)
pRS423	2μ ori *HIS3*	(Sikorski and Hieter, 1989)
pRS425	2μ ori *LEU2*	(Sikorski and Hieter, 1989)
pXP346	*can1Δ::LEU2-MFA1*$_{pr}$*-HIS3*	(Pan *et al.*, 2004)
pUD193	*CEN6-ARS4*	Genescript
pUD195	*E. coli* replication origin	Genescript
pUDC071	2μ *URA3* $ARO10_{pr}$-*yEGFP-CYC*$_{ter}$	This study
pUDE004	2μ *URA3* $TDH3_{pr}$-$ARO7^{G141S}$-CYC_{ter}	(Luttik *et al.*, 2008)
pUDCTAR	2μ *HIS3 MET15 LEU2*	This study

homologous flanking regions (770 bp) was amplified from genomic DNA of strain IMK552 (*aro8Δ*::*amdSYM*) using Phusion Hot Start polymerase (Finnzymes, Landsmeer, The Netherlands) with primers ARO8 big del RV/ARO8 F. The resulting 4 kb PCR product was gel-purified and used to transform strain IMK431 with the lithium acetate method and plated on selective medium containing acetamide, resulting in strain IMK559 (*aro8Δ*::*amdSYM aro80Δ*::*kanMX*). To confirm deletion of *ARO8*, primers ARO8 E/Amds Rv were used. To construct the strain IMC063, the fragments that composed the plasmid pUDCTAR were assembled by *in vivo* recombination in BY4741 (Kuijpers *et al.*, 2013). The *LEU2*, *MET15*, and *HIS3* genes were PCR amplified using the primer pairs LEU2A/LEU2F, MET15G/MET15H and HIS3B/HIS3I and the plasmid templates pRS425 (*LEU2*), pRS411 (*MET15*) and pRS423 (*HIS3*), respectively. The fragments containing the amp^r gene and the *Escherichia coli ori* sequence were obtained by digesting the plasmid pUD193 with SacII while the *CEN6-ARS4* region was cut out of plasmid pUD195, digesting with *Not*I. DNA fragments were gel-purified and transformed in strain BY4741, using the lithium acetate method (Gietz and Woods, 2002). Transformants were selected on synthetic solid medium supplemented with uracil. Plasmid pXP346 (Pan *et al.*, 2004) carried the *LEU2* marker associated to *HIS3* under the control of the *MFA1* promoter, allowing the expression of the *HIS3* gene exclusively in *MAT***a** strains. The plasmid was digested with *Spe*I and *Pst*I and the resulting 4.6 kb fragment, that included flanking sequence to guide integration at the *CAN1* locus, was gel-purified and used to transform strain BY4742 (*MATα*) resulting in strain IMX100. The correct strain was selected on synthetic solid medium plates supplemented with 60 mg/l canavanine, 75 mg/l lysine, 125 mg/l histidine and 150 mg/l uracil.

To construct plasmid pUDC071, a 718 base-pair (bp) fragment comprising the *ARO10* promoter was PCR-amplified from genomic DNA of *S. cerevisiae* CEN.PK113-7D with primers ARO10 pr Fw *Sac*I/ARO10 pr Rv *Hin*dIII. The product was digested with *Sac*I and *Hin*dIII and ligated into pAG416GAL-ccdB *yEGFP* digested with the same enzymes. The ligation mixture was transformed into *E. coli* DH5*α*. After selection on ampicillin, the presence of the plasmid was confirmed using primers Aro10 KO CHK/FK098 and restriction analysis. This plasmid was subsequently transformed into strains CEN.PK113-5D, IMX100, IMY065, IMY067, IMY070, IMC063, IMY092, IMK525, IMK552, IMK431, IMK497 and IMK559, resulting in strains IME185, IMX101, IMY072, IMY074, IMY078, IMY079, IMY093, IMZ461, IMZ437, IMZ430, IMZ432 and IMZ458, respectively.

Synthetic genetic-array analysis (SGA)

To introduce the yeast enhanced green fluorescent protein (*yEGFP*)-based reporter construct pUDC071 into all haploid *S. cerevisiae* strains carrying deletions in individual non-essential genes, the synthetic genetic array (SGA) method was used (Figure 1) (Tong *et al.*, 2001). Each haploid *MAT***a** strain in the *S. cerevisiae* deletion strain collection carries a

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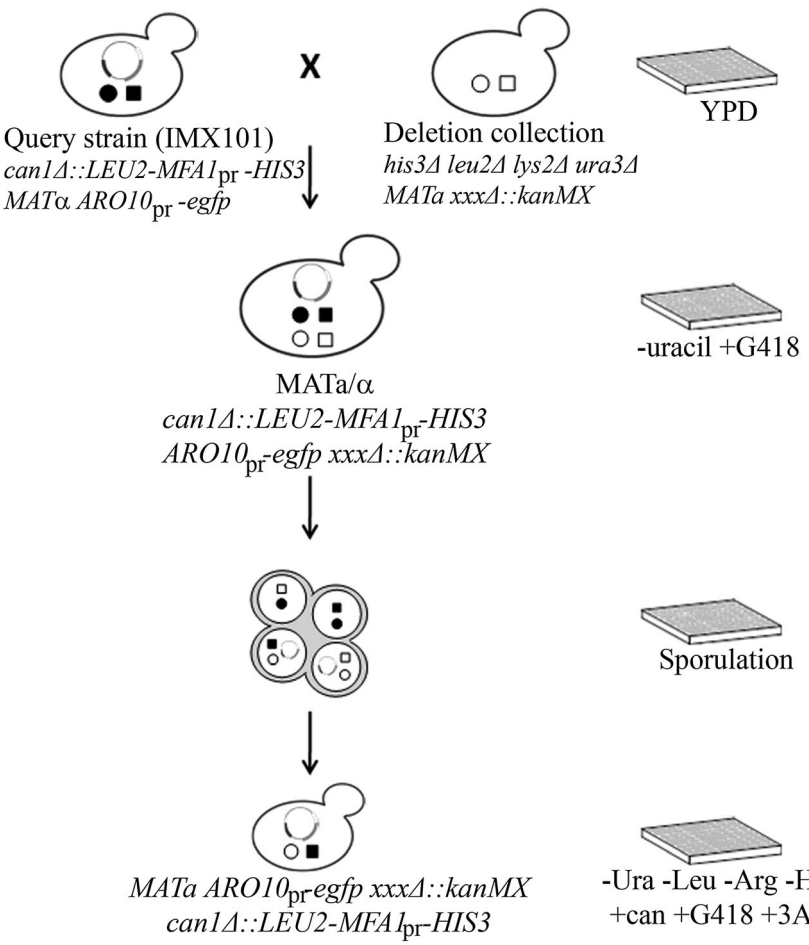

Figure 1. Schematic overview of the SGA procedure. A *MATα* strain carrying the reporter plasmid pUDC071 (URA3 $ARO10_{pr}$–*yEGFP*) and the chromosomal insertion of the mating type dependent marker MFA_{pr}–*HIS3* was crossed to an ordered array of *MATa* viable yeast deletion mutants carrying a gene deletion (*xxxΔ::kanMX*). Selection of diploids was done on solid medium lacking uracil and supplemented with G418. The diploids are transferred first on sporulation medium and afterward on medium for selection of haploid *MATa* strains carrying both the deletion and the reporter plasmid

single gene replacement with the *kanMX* selectable cassette (Giaever *et al.*, 2002). The haploid *MATα* strain IMX101 (referred to as query strain) carries: (a) the *CAN1L-LEU2-MFA1pr-HIS3-CAN1R* selectable cassette that used *LEU2* to select for the presence of the chromosomal integration of the cassette at the *CAN1* locus and the expression module $MFA1_{pr}$*-HIS3* for the specific selection of haploid *MAT***a** segregants; and (b) the pUDC071 plasmid harbouring the reporter construct (Figure 2). All media required for the SGA procedure are described in Table S1 (see supporting information). SGA was performed using a Rotor HAD robot (Singer Instruments, Somerset, UK). The query strain was initially grown in YNB supplemented with uracil. The culture was put into a sterile square container and, using a 384-pin replicator, the liquid was transferred into a new YNB plate lacking uracil and incubated at 30°C for 2 days, generating a pool of cells for the following mating step. The yeast deletion collection (YDC) was replicated in a 384 plate format and grown on YPD plates supplemented with G418 for 1 day. The 384-format *MATα* query strain plate and the 384 plate format *MAT***a** YDC were pinned on fresh YPD plate for mating. The resulting *MAT***a**/*α* diploids were transferred onto YNB plates without uracil, to select for strains carrying the plasmid, and supplemented with G418, to select for the deletion of interest. After incubation for 2 days at 30°C the diploids were transferred onto sporulation plates for

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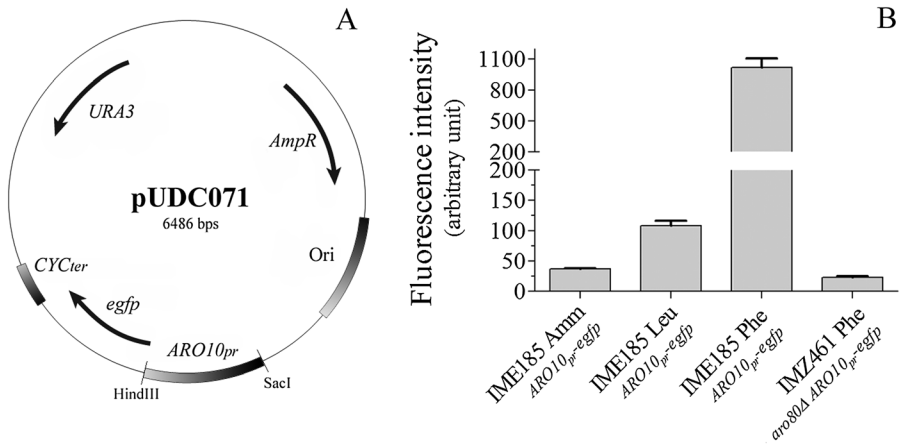

Figure 2. Validation of the reporter construct. (A) Map of plasmid carrying the *ARO10*$_{pr}$–*yEGFP* construct (B) Average fluorescence of strain IME185 (*ARO10*$_{pr}$–*yEGFP*) and IMZ461 (*aro80Δ ARO10*$_{pr}$–*yEGFP*) on ammonium (Amm), leucine (Leu) or phenylalanine (Phe) as sole nitrogen sources. Cells were grown in shake flasks on synthetic medium with glucose and ammonium sulphate and harvested during exponential growth to measure fluorescence using flow cytometry. Reported values are averages and SDs of at least three independent replicates

9 days at 22°C. The *MAT***a** haploid strains carrying the pUDC071 plasmid and the deletion of interest were transferred and selected for 2 days on YNB lacking uracil, leucine, arginine and histidine and supplemented with G418, canavanine and 3-aminotriazole. This last selection step was repeated twice to ensure strain purity. Finally, colonies were retransferred to 96-well plates and grown overnight on YNB liquid medium without uracil supplemented with G418, prior to the addition of 50% glycerol and storage at −80°C.

Flow cytometry and data analysis

For screening of *yEGFP* expression, a 96-pin replicator was used to inoculate strains into 96-well plates containing synthetic glucose medium with ammonium sulphate as sole nitrogen source. Plates were covered with a gas-permeable seal in order to allow gas transfer and incubated for at least 16 h at 30°C in an Innova incubator shaker (250 rpm; New Brunswick Scientific, NJ, USA). Flow cytometry was conducted using a Quanta flow cytometer system (Beckman, Woerden, The Netherlands). For each of the 4582 unique gene deletion strains tested, 1000 events were collected. Scatter and fluorescence measurements were recorded on a range of 1024 values, i.e. 0–1023. These raw data were processed to eliminate instrument errors and outliers, as described previously (Newman *et al.*, 2006). Additionally, events with a forward scatter < 350 and/or fluorescence values 200 were assumed to represent debris or dead cells and were discarded (Figure 3A). Scatter and fluorescence values were normalized across plates by scaling the robust mean of these measurements to the same value for each plate. Compensation of fluorescence values for differences in cell morphology (as measured by forward and side scatter) were computed as described previously (Knijnenburg *et al.*, 2011). Two outlier tests were applied to identify strains with significantly higher or lower fluorescence than the bulk of strains. Two data vectors were created; one that contained the mean fluorescence values for each strain, $\boldsymbol{x}$, and one that contained the percentage of fluorescent cells (fluorescence > 200) for each strain, $\boldsymbol{y}$. The same test was applied to both data vectors. This test modelled the values in a vector as an asymmetric normal distribution with a mean equal to the mode of the values, the left standard deviation (SD) equal to the 68% percentile of the deviations from the mode of the values smaller than the mode, and the right SD equal to the 68% percentile of the deviations from the mode of the values larger than the mode. The distribution of mean fluorescence values for each strain in $\boldsymbol{x}$ and the asymmetric normal model is depicted in Figure 3B. Left- and right-tailed p values were derived from this normal distribution for each strain to identify strains with significantly larger or smaller values than the bulk of strains, respectively. The two p values (one for each test) were combined using Fisher's method. The 43 strains (with

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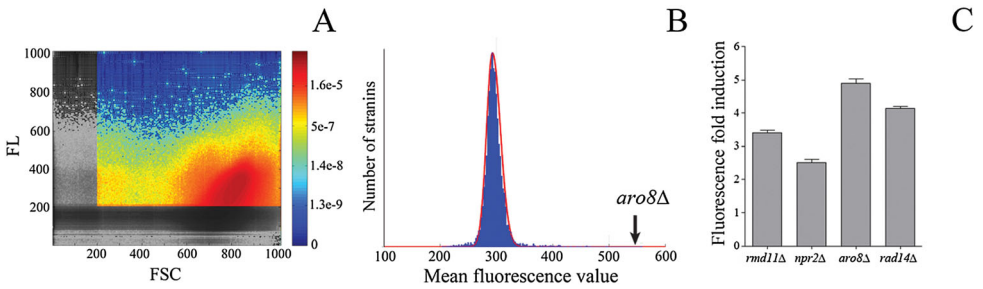

Figure 3. Screening of yeast deletion collection carrying the *ARO10*$_{pr}$–*yEGFP* construct. (A) Cells were grown on a 96-well plate for 16 h, and subsequently measured by flow cytometry. Fluorescence (FL) and forward scatter (FSC) raw data of each cell measured are reported as a density plot. The grey region was assumed to contain debris or dead cells, which were subsequently removed from the analysis by gating. (B) Histogram of the means of the normalized fluorescence values for each strain and fitted normal distribution for *p* value calculation. (C) Strains with $p < 10^{-4}$ resulting directly from the SGA were rescreened in quadruplicate and the results for the confirmed mutations were expressed as fold induction compared to the reference strain BY4741

at least 200 cells after gating for debris and dead cells) with a combined *p* value $< 10^{-4}$ were selected for a second screening. Flow-cytometry data for all strains, including the fluorescent mean, percentage of fluorescent cells and the individual and combined *p* values, are included in Table S2 (see supporting information). During a second screen, each strain was measured in quadruplicate and for each well 10 000 cells were analysed.

Transcriptome data analysis

Sampling of cells from chemostat cultures and total RNA extraction was performed as described previously (Hazelwood *et al.*, 2009; Knijnenburg *et al.*, 2009). Probe preparation and hybridization to Affymetrix Genechip® microarrays were performed according to the manufacturer's instructions. The one-cycle eukaryotic target labelling assay was used, starting with 15 µg total RNA. The quality of total RNA, cRNA and fragmented cRNA was checked using the Agilent BioAnalyzer 2100 (Agilent Technologies, Amstelveen, The Netherlands). For each strain, results were obtained from two independent culture replicates. The Significance Analysis of Microarrays (SAM v 1.12; Tusher *et al.*, 2001) add-in to Microsoft Excel was used for comparison of replicate array experiments, using a fold-change threshold of 2 and a false-discovery rate of 3.4%. Transcript data generated in this study have been deposited in the Genome Expression Omnibus database under Accession No. GSE52256. Chemostat-based transcriptome data for strain CEN.PK113-7D grown with either ammonium sulphate or phenylalanine as sole nitrogen source were obtained from the Genome Expression Omnibus database series number GSE6405 (Boer *et al.*, 2007; Knijnenburg *et al.*, 2009).

Intracellular metabolites analysis

For intracellular metabolite measurements, chemostat cultures were rapidly sampled and quenched in 80% methanol at a temperature of –40ºC (Canelas *et al.*, 2008); 800 µl samples were added directly to 5 ml tubes of precooled methanol. Subsequently, cold methanol was used to wash the samples by centrifugation and the metabolites were extracted with boiling ethanol, as described previously (Canelas *et al.*, 2009). Accurate intracellular metabolite quantification was based on addition of an internal standard in the form of uniformly labelled ^{13}C-cell extract (Wu *et al.*, 2005). Using GC–MS, the concentration and mass shift of ^{13}C samples was measured for pyruvate (Pyr), alanine (Ala), glycine (Gly), valine (Val), leucine (Leu), iso-leucine (Ile), proline (Pro), serine (Ser), threonine (Thr), methionine (Met), aspartate (Asp), phenylalanine (Phe), cysteine (Cys), glutamate (Glu), lysine (Lys), asparginine (Asp), glutamine (Gln), tyrosine (Tyr), histidine (His) and tryptophan (Trp), using a previously described protocol (van Dam *et al.*, 2002).

Results

Genetic screening of factors involved in regulation of *ARO10*

To measure activity of the *ARO10* promoter in individual deletion strains, we constructed a *yEGFP*-

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based reported construct in which the *ARO10* promoter was fused to the *yEGFP* coding sequence (Figure 2A). To test whether yEGFP fluorescence could be used as a reliable readout of *ARO10* promoter activity, strain IME185 (*ARO10$_{pr}$-yEGFP-CYC1$_{ter}$*) was grown on glucose with different nitrogen sources (ammonium, leucine and phenylalanine), followed by analysis of fluorescence with flow cytometry. Growth on phenylalanine resulted in 30-fold higher fluorescence levels than growth on ammonium. Consistent with previous data (Boer *et al.*, 2007), leucine partially induced the *ARO10* promoter, leading to fluorescence levels that were three-fold higher than those in ammonium-grown cultures (Figure 2B). To test whether phenylalanine-induced fluorescence was dependent on Aro80, the only known transcriptional activator of *ARO10*, *ARO80* was deleted in strain IME185 (*ARO10$_{pr}$–yEGFP*), resulting in strain IMZ461 (*ARO10$_{pr}$–yEGFP aro80Δ*). Indeed, fluorescence in phenylalanine-grown cultures of the latter strain was much lower than in strain IME185 and close to the level observed in ammonium-grown cultures. These results validated the functionality of the *ARO10$_{pr}$–yEGFP* reporter construct, thus paving the way for its application in a genetic screen for genes involved in transcriptional (de) repression of *ARO10*.

A 96-well plate-arrayed collection of strains which combined the *ARO10$_{pr}$–yEGFP* reporter construct with individual deletions in non-essential yeast genes was constructed by Synthetic Genetic Array (SGA) technology (Tong *et al.*, 2001). To do so, the query strain IMX101 (*MATα his3Δ1 leu2Δ lys2Δ ura3Δ can1::LEU2-MFA1pr-HIS3::can1* pUDC071 [2μ *URA3 ARO10$_{pr}$-yEGFP - CYC$_{ter}$*]) was initially crossed to an ordered array of viable gene deletion mutants (*MAT***a** *his3Δ1 leu2Δ met15Δ ura3Δ xxxΔ::kanMX*). Diploid strains containing the mutation of interest and the plasmid with the reporter system were selected on agar plate containing G418 and lacking uracil. After sporulation, only *MAT***a** strains were selected on agar plate without histidine and supplemented with canavanine. The newly 96-well plate arrayed collection which combined the *ARO10$_{pr}$–yEGFP* reporter construct and individual single gene deletions was grown on medium containing ammonium as sole nitrogen source. After overnight growth in shaken 96-well plates, the fluorescence of 1000 cells of each SGA-derived strain was measured by flow cytometry. For quantitative analysis, a gate was imposed on the FL/FSC two-dimensional space (Figure 3A), followed by plate normalization and a correction for cell morphology using a regression model (Knijnenburg *et al.*, 2011). The final normalized fluorescence data were plotted in a histogram and fitted with a normal distribution for the identification of outliers (Figure 3B). When the 43 strains with a p value $< 10^{-4}$ were retested in quadruplicate, a confirmation of the initial screening results was obtained for only four strains (Figure 3C). These four strains carried deletions in the following genes: (a) *RAD14,* encoding a subunit of the nucleotide excision repair factor that recognizes and binds damaged DNA; (b) *NPR2* and *RMD11*, encoding two components of a complex that mediates the amino acid starvation signal to TORC1; and (c) *ARO8*, encoding one of the aromatic amino acid aminotransferases in *S. cerevisiae*.

Verification of SGA results

To verify the genotype of the strains constructed via the high-throughput SGA approach, the four 'hits' identified by flow-cytometry screening were subjected to PCR analysis with gene-specific primers pairs flanking the deletion cassette. This analysis confirmed that each of the four strains carried the expected deletion. To investigate potential effects of strain background and/or auxotrophy, the four deletions were each introduced in *S. cerevisiae* CEN.PK113-5D (Entian and Kötter, 2007) as a host. Furthermore, the four strains were reconstructed in the BY4741 background. The newly constructed deletion strains were then transformed with pUDC071 and the BY4741-derived deletion strains were co-transformed with the pUDCTAR plasmid. Upon screening of the resulting prototrophic strains by flow cytometry, only the *aro8Δ* strains in the two genetic backgrounds showed an eight-fold increase of yEGFP fluorescence under ammonium-repressed conditions relative to the fluorescence of the corresponding reference strains (Table 4). Under these conditions, fluorescence of the strains deleted in *RAD14*, *NPR2* and *RMD11* was not different from that of the control strain IMY079 and IME185 (Table 4). This result left the *ARO8* deletion as the sole mutation that consistently resulted in increased transcription of the *ARO10* promoter, independent of genetic background and prototrophy of the host strain. To verify whether transcriptional activity of

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Table 4. Induction of the *ARO10$_{pr}$–yEGFP* reporter construct in different genetic backgrounds

Background	Strain	Relevant genotype	Fluorescence (arbitrary unit)	
			Ammonium	Phenylalanine
BY4741	IMY079	Reference strain	29 ± 0.8	260 ± 18
	IMY078	*npr2Δ*	23 ± 0.2	250 ± 17
	IMY093	*rmd11Δ*	21 ± 0.7	261 ± 23
	IMY065	*aro8Δ*	176 ± 5.1	253 ± 20
	IMY067	*aro80Δ*	16 ± 1.2	21 ± 1.2
CEN.PK113-5D	IME185	Reference strain	26 ± 1.2	230 ± 19
	IMZ430	*npr2Δ*	20 ± 0.9	240 ± 15
	IMZ432	*rmd11Δ*	19 ± 1.3	250 ± 12
	IMZ437	*aro8Δ*	165 ± 8	260 ± 22
	IMZ461	*aro80Δ*	15 ± 1.5	23 ± 2.6
	IMZ458	*aro8Δ aro80Δ*	16 ± 0.9	19 ± 2.9

Strains were grown on glucose synthetic medium with ammonium or phenylalanine as sole nitrogen source. Samples were taken during exponential growth phase and fluorescence was measured with flow cytometry. Results are reported as average ± SD of three independent biological replicates.

the *ARO10* promoter was still Aro80-dependent (Iraqui *et al.*, 1999) in the *aro8Δ* background, strain IMZ458 (*aro8Δ aro80Δ*) was constructed and its fluorescence analysed under ammonium-repressed conditions. A significant reduction of the yEGFP fluorescence (Table 4) relative to the *aro8Δ* reference strain demonstrated that expression of the *ARO10* promoter was still under the control of the transcriptional activator Aro80, despite the absence of added aromatic amino acid inducer.

Characterization of an *aro8Δ* strain in chemostat cultures

To quantitatively analyse the impact of the *ARO8* deletion, *S. cerevisiae* IMY081 *(aro8*Δ) and its isogenic reference strain CEN.PK113-7D were grown in glucose-limited chemostat cultures. Chemostat cultivation is a highly reproducible system for metabolomics and genome-wide expression analysis which enables the quantitative assessment of individual environmental parameters or specific genetic modifications at a fixed, controllable specific growth rate (Daran-Lapujade *et al.*, 2009). In aerobic, glucose-limited chemostat cultures grown at a specific growth rate of 0.10 h^{-1}, physiological analysis of strains IMY081 and CEN.PK113-7D revealed two significant differences (Table 5). IMY081 exhibited a slightly lower biomass yield than CEN.PK113-7D (0.47 ± 0.01 vs 0.50 ± 0.01 g/g) and, in contrast to the reference cultures, in which phenylethanol and phenylacetate were not detectable, a combined flux of 4.7 μM/g/h phenylethanol and phenylacetic acid was observed. The low concentrations of phenylacetate (0.06 mM) may have contributed to the lower biomass yield of strain IMY081 by weak-acid uncoupling of the plasma-membrane pH gradient and ATP-driven export of phenylacetate via Pdr12 (Hazelwood *et al.*, 2006).

To further investigate the mechanism by which the *ARO8* deletion deregulates *ARO10* promoter activity, microarray analyses were performed on chemostat cultures of strains IMY081 and CEN.PK113-7D. The average coefficient of variation of the replicates for both strains was < 18% and statistical analysis revealed that 52 and 113 genes showed higher or lower (fold difference > 2) transcript levels in the deletion strain, respectively (see supporting information, Table S3). Enrichment analysis with a Fischer exact test did not yield any over-represented functional categories among these genes. As expected, *ARO8* expression was completely abolished in IMY081. However, the *ARO9* gene, which also encodes an aromatic amino acid transaminase, was not differently expressed (Iraqui *et al.*, 1998) and, in contrast with previously reported data (Iraqui *et al.*, 1998, 1999), was already highly expressed in the ammonium-grown cultures of the reference strain CEN.PK113-7D. Consistent with the results of the genetic screen, expression of *ARO10* was significantly (2.1-fold) higher in strain IMY081 than in the reference strain. The difference in induction amplitude of the yEGFP reporter construct (eight-fold) and transcript levels (2.1-fold) may be due to differences in cultivation conditions (shake flask vs chemostat

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Table 5. Physiology of *Saccharomyces cerevisiae* strains IMY081 (*aro8Δ*) and CEN.PK113-7D (reference) in aerobic glucose-limited chemostat cultures at a dilution rate of 0.1 h^{-1}

	IMY081 (*aro8Δ*)	CEN.PK113-7D (reference)
Biomass yield (g/g glucose)	0.47 ± 0.01	0.50 ± 0.00
CO_2 production rate (mM/g dw/h)	2.6 ± 0.2	2.8 ± 0.1
Glucose consumption rate (mM/g dw/h)	−1.15 ± 0.04	−1.10 ± 0.01
Glycerol (mM)	0.51 ± 0.12	–
PheEtOH (mM)	0.07 ± 0.02	–
Phe acetate (mM)	0.08 ± 0.02	–
OH-PheEtOH (mM)	–	–

Values represent the average ± mean deviation of biological duplicates.
–, absence of detectable concentrations of the metabolite in a culture supernatant.

cultures) or to the target gene copy number (episomal plasmid vs chromosomal gene) or to more likely the transcript's stability and translation efficiency (changes in 5′ or 3′ end distributions) (Fehrmann *et al.*, 2013).

While the changes in transcript levels were modest, intracellular metabolite analysis revealed a major impact of the deletion of *ARO8* on intracellular amino acid pools. With the exception of cysteine, all other amino acids showed higher intracellular concentrations in *S. cerevisiae* IMY081 (*aro8*Δ) than in the reference strain CEN.PK113-7D. The intracellular concentrations of the aromatic amino acids phenylalanine, tryptophan and tyrosine were 2.1-fold, 1.9-fold and 1.2 fold higher in the deletion mutant, respectively. Strikingly, the largest increases were observed for threonine (3.2-fold) and alanine (3.1-fold). The overall intracellular amino acid concentration in the *aro8*Δ strain was 2.3-fold higher than in the reference strain (Figure 4).

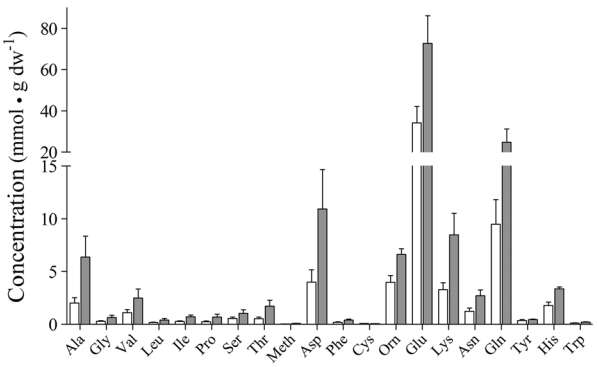

Figure 4. Impact of *ARO8* deletion on intracellular amino acid concentrations. Strains CEN.PK113-7D (reference strain; white bars) and IMY081 (*aro8Δ*; grey bars) were grown in aerobic glucose-limited chemostat cultures. Once steady state was reached, a sample was taken for intracellular metabolite analysis. The results are expressed in mM/g dry weight and represent the average and mean deviation of independent biological duplicates

Engineering *de novo* phenylethanol biosynthesis in an *S. cerevisiae aro8Δ* mutant

Previous studies on phenylethanol production by *S. cerevisiae* mostly focused on bioconversion of extracellularly added phenylalanine into phenylethanol (Cui *et al.*, 2011; Eshkol *et al.*, 2009; Etschmann *et al.*, 2005; Kim *et al.*, 2014). The data discussed above suggest that an *aro8*Δ mutation may be useful for *de novo* production of phenylethanol from glucose in ammonium-containing media. Consistent with the chemostat data (Table 5), deletion of *ARO8* was sufficient to cause detectable production of phenylethanol in shake-flask cultures of strain IMY081 (Figure 5). In contrast to the observations in chemostat cultures, *p*-hydroxyphenylethanol, the higher alcohol derived from tyrosine, was also detected in the shake-flask culture supernatants (Figure 5). The total amount of aromatic higher alcohols (*p*-hydroxyphenylethanol and phenylethanol) produced by the *aro8*Δ mutant (IMY081) was comparable to that in the congenic strain *S. cerevisiae* IMN002, which expresses phenylalanine and tyrosine feedback-insensitive DAHP synthase (*aro3*Δ *ARO4*^{K229L}↑) (Luttik *et al.*, 2008). Combination of these mutations in a single strain resulted in a further increase in aromatic alcohol production, reaching a total concentration of 2.8 mM (strain IMN013, Figure 5). Similarly, the combination of the *ARO8* deletion with the overexpression of feedback-insensitive DAHP synthase (*ARO4*^{K229L}) and chorismate mutase (*ARO7*^{G141S}) alleles (IMN014) resulted in increased higher aromatic

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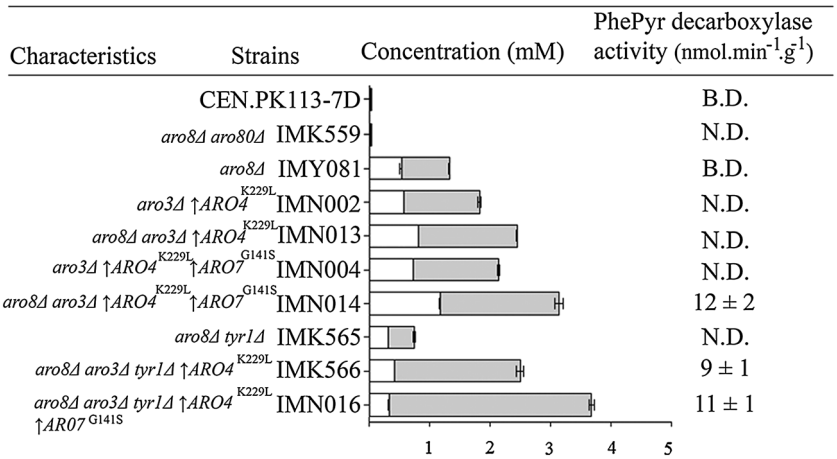

Figure 5. Combination of *ARO8* deletion with other mutations: impact on aromatic alcohol production. *S. cerevisiae* strains carrying different combinations of mutations that affect aromatic amino acid metabolism were grown on glucose synthetic medium with ammonium as the sole nitrogen source and supplemented with 0.4 mM tyrosine when required. After reaching stationary phase, samples were taken for extracellular metabolite analysis. White and grey bars represent average concentration ± SD of *p*-hydroxyphenylethanol and phenylethanol, respectively, of three independent replicates. Enzymatic activity measurements are reported as average ± mean deviation of technical duplicates of one of the triplicated cultures. B.D., below detection limit (estimated at 4 nmol/min/mg protein); N.D., samples for which the enzymatic activity was not determined

alcohol concentrations relative to those in cultures of a strain only overexpressing the two feedback-insensitive alleles (IMN004).

The formation of tyrosine as a by-product should be prevented by deletion of *TYR1*, which encodes prephanate dehydrogenase. Since *tyr1*Δ strains are tyrosine auxotrophs a low concentration of tyrosine (0.4 mM) was added to growth media. Combination of the *ARO8* and *TYR1* deletions (IMK565) resulted in a reduced production of both aromatic alcohols, possibly as a consequence of tyrosine inhibition on the DAHP synthase (Helmstaedt *et al.*, 2001; Krappmann *et al.*, 2000; Kunzler *et al.*, 1992) and chorismate mutase (Krappmann *et al.*, 2000) encoded by wild-type *ARO4* and *ARO7* alleles, respectively (Figure 5). To fully investigate the impact of the prephenate dehydrogenase deletion on *p*-hydroxy- and phenylethanol formation, the genes encoding the DAHP synthase activity were altered; *ARO3*, which encodes a phenylalanine feedback-inhibited allele, was deleted, and the wild-type *ARO4* was replaced by the $ARO4^{K229L}$ that encodes a tyrosine feedback-insensitive DAHP synthase allele (Luttik *et al.*, 2008). The subsequent strain IMK565 (*aro8Δ tyr1Δ aro3Δ* $ARO4^{K229L}$↑) showed a 2.5-fold reduction in *p*-hydroxyphenylethanol relative to IMN013 (*aro8Δ aro3Δ* $ARO4^{K229L}$↑) (Figure 5). The residual concentration (0.30 mM) presumably originated from the bioconversion of the supplemented tyrosine.

Similarly the wild-type *ARO7* allele was replaced by the tyrosine feedback insensitive $ARO7^{G141S}$ allele and combined with the *aro8*, *tyr1*, *aro3* mutations and $ARO4^{K229L}$ overexpression. The resulting strain IMN016 (*aro8*Δ *tyr1*Δ *aro3*Δ $ARO4^{K229L}$↑ $ARO7^{G141S}$↑) produced up to 3.34 mM phenylethanol and only 0.33 mM *p*-hydroxyphenylethanol (Figure 5). Low but significant phenylpyruvate decarboxylase activities were measured in cell extracts of strains IMN014, IMN016 and IMK566. This is remarkable, because phenylpyruvate decarboxylase activity has not been detected in cell extracts of wild-type *S. cerevisiae* grown under ammonium-repressed conditions (Romagnoli *et al.*, 2012).

Discussion

Interpretation of genome-wide screen for factors involved in regulation of *ARO10*

The present study was based on a genome-wide screen for *S. cerevisiae* genes involved in the (de) repression, in the presence of ammonium, of *ARO10*, which encodes the major decarboxylase involved in phenylethanol production by *S. cerevisiae* (Romagnoli *et al.*, 2012; Vuralhan

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et al., 2005).The screen yielded only *ARO8* as a confirmed 'hit' but, for several reasons, this result does not exclude involvement of additional, as yet unidentified, genes in the transcriptional regulation of *ARO10*. First, the high incidence of false positives in the initial screen may also suggest occurrences of missed false negatives. While there is no necessary general correlation, some examples support this idea (Askree *et al.*, 2004; Gatbonton *et al.*, 2006). Second, a screen of single-deletion mutants cannot identify deletions whose impact depends on the genetic interactions with other genes. The relevance of this caveat for the present study is demonstrated by an earlier observation on tryptophan-mediated activation of *ARO9* and *ARO10* by GATA factors Gat1 and Gln3, which bind the *ARO9* and *ARO10* promoters in the presence of rapamycin (Lee and Hahn, 2013). This activation is only abolished in a *gln3Δ gat1Δ* double mutant, but not in the corresponding single-deletion mutants (Iraqui *et al.*, 1999). Third, three confirmed 'hits' from the initial screen, which was carried out in an auxotrophic strain background, did not influence *ARO10* expression when the corresponding deletions were retested in prototrophic strain backgrounds. The latter result confirms the importance of taking into account the impact of auxotrophic markers in physiological studies (Pronk, 2002).

The impact of the *aro8*Δ mutation on *ARO10* expression was demonstrated in two distinct genetic backgrounds, indicating that it is not an atypical characteristic of a single *S. cerevisiae* strain. However, our chemostat-based transcriptome analyses did suggest a strain-dependent regulation of *ARO9*, a paralogue of *ARO8* that encodes an inducible aromatic amino acid amino transferase (Godard *et al.*, 2007; Iraqui *et al.*, 1998). In other strain backgrounds, such as ∑1278b or BY4741 (Iraqui *et al.*, 1999), *ARO9* has been shown to be tightly co-regulated with *ARO10*, with Aro80-mediated transcription of both genes being tightly dependent on the presence of an aromatic amino acid inducer. In contrast, our results with strain CEN.PK113-7D showed a high basal expression level of *ARO9* in cultures grown with ammonium sulphate as the nitrogen source (ca. two-thirds of the expression level of its constitutively expressed paralogue *ARO8*; Figure 6). Furthermore, although *ARO9* was clearly induced in phenylalanine-grown cultures (by three-fold relative to ammonium-grown

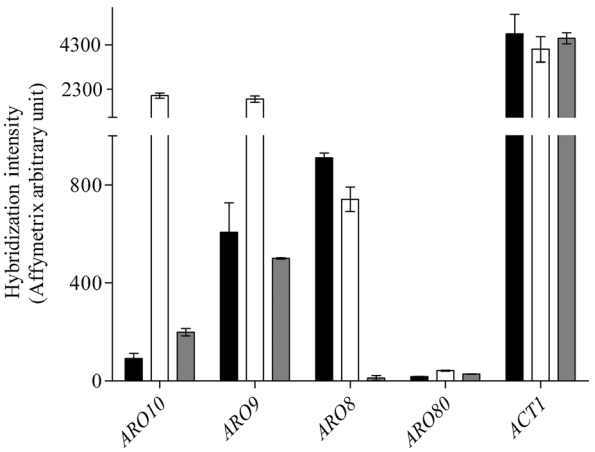

Figure 6. Impact of *ARO8* deletion on transcription of Aro80 target genes. Transcript levels of *ARO9, ARO10, ARO80, ARO8* and *ACT1* in aerobic, glucose-limited chemostat cultures of *S. cerevisiae* CEN.PK113-7D grown with $(NH_4)_2SO_4$ (black bar) and phenylalanine (white bar) and IMY081 (*aro8*Δ) grown with $(NH_4)_2SO_4$ (grey bar). Transcript levels were determined with Affymetrix GeneChips YG-S98 and are the averages of results from at least two independent experiments. Data for strain CEN.PK113-7D grown with $(NH_4)_2SO_4$ (black bar) and phenylalanine (white bar) were obtained from the Genome Expression Omnibus database series number: GSE 6405 (Boer *et al.*, 2007; Knijnenburg *et al.*, 2009)

cultures), the amplitude of this induction was much lower than observed for *ARO10* (22-fold; Figure 6). This result extends previous observations that important aspects of the regulation of nitrogen metabolism in *S. cerevisiae* can be strain background-dependent (Georis *et al.*, 2009).

Roles of Aro8 and Aro80 in transcriptional regulation of *ARO10*

Since *ARO8* encodes a well-characterized transaminase without a known role in transcriptional regulation (Bulfer *et al.*, 2013; Iraqui *et al.*, 1998; Urrestarazu *et al.*, 1998), its involvement in derepression of *ARO10* was unexpected. In wild-type strains, induction of *ARO10* depends on the presence of an aromatic amino acid in the culture medium (Chen and Fink, 2006; Dickinson *et al.*, 2003; Prusty *et al.*, 2004; Vuralhan *et al.*, 2005). Although not experimentally proven, it is generally assumed that physical interaction of an aromatic amino acid with Aro80 is required for its activation (Iraqui *et al.*, 1999; Lee and Hahn, 2013). In our study, increased expression of *ARO10* in *aro8Δ*

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S. cerevisiae grown under ammonium-repressed conditions was dependent on the presence of a functional *ARO80* gene. Our results are therefore consistent with a model in which *ARO8* deletion causes a change in the intracellular concentration of (a) low-molecular-weight effector(s) of Aro80, whose activation subsequently causes increased expression of *ARO10*. This hypothesis is further supported by the increased intracellular concentrations of amino acids, including the aromatic amino acids phenylalanine, tyrosine and tryptophan, in an *aro8Δ* strain.

Impact of *ARO8* deletion on phenylethanol production and intracellular amino acid pools

Deletion of *ARO8* was shown to stimulate production of phenylethanol in a wild-type background and when the *aro8Δ* mutation was combined with other mutations that had previously been demonstrated to stimulate production of this industrially relevant compound. As discussed above, identification of *ARO8* as a target gene for engineering of phenylethanol production was based on a screen for factors involved in transcriptional deregulation of *ARO10*. However, this deregulation is not necessarily the only mechanism by which the *aro8Δ* mutation affects phenylethanol production. In a previous study, expression of *ARO10* from a strong constitutive promoter did not lead to increased phenylpyruvate decarboxylase activity under ammonium-repressed conditions (Vuralhan *et al.*, 2005). However, using the same expression construct, activity was observed when phenylalanine was the nitrogen source, suggesting that Aro10p is regulated post-translationally (Romagnoli *et al.*, 2012). Interestingly Aro10 was identified as a target for ubiquitination, suggesting a possible regulation mechanism (Peng *et al.*, 2003). In the present study, low but significant activities of phenylpyruvate decarboxylase were measured in several *aro8Δ* strains grown with ammonium sulphate as sole nitrogen source, suggesting that the changes in intracellular amino acid pools caused by deletion of *ARO8* not only stimulated Aro80-mediated expression of *ARO10*, but also enabled post-translational activation of the Aro10 protein.

The present study revealed that elimination of a single transaminase gene in *S. cerevisiae* can have a strong impact on intracellular amino acid concentrations. The impact of the *aro8Δ* deletion extended beyond the known aromatic substrates of Aro8 and even affected the intracellular concentrations of almost all proteinogenic amino acids. Transaminases are ubiquitous enzymes that participate in both amino acid biosynthesis and amino acid catabolism by the transfer of amino groups between amino donors and 2-oxo acid acceptors. The *S. cerevisiae* genome harbours no fewer than 18 annotated transaminase genes, of which 10 form paralogue pairs (*AAT1/AAT2*, *ALT1/ALT2*, *BAT1/BAT2*, GFA1/YMR084W-YMR085W and *ARO8/ARO9*). Even the transaminase paralogues can have different kinetic properties and substrate specificities. Kradolfer *et al.* (1982) showed that Aro8 has a lower K_m for phenylalanine and tyrosine ($K_m = 0.3$ mM) than for tryptophan ($K_m = 6$ mM), whereas in Aro9 K_m values for these three amino acids were similar (0.2–0.4 mM) (Kradolfer *et al.*, 1982). The different substrate specificities of Aro8 and Aro9 are exemplified by the observation that Aro9 does not accept glutamate as an amino donor and exhibits a very low affinity for 2-oxoglutarate (Iraqui *et al.*, 1998; Urrestarazu *et al.*, 1998), implying that it cannot effectively use the most abundant amino donor/acceptor couple in yeast cells.

The inherent reversibility of the transaminase reactions and the different, but overlapping substrate specifity of the *S. cerevisiae* transaminases already make it difficult to predict the outcome of genetic interventions that affect expression of single transaminases. Experimental analysis and modelling of the transaminases is further complicated by the compartmentation of intracellular amino acid pools, which involves separate cytosolic, mitochondrial and vacuolar pools (Kitamoto *et al.*, 1988; Szabados and Savouré, 2010). The size and composition of intracellular amino acid pools is highly relevant for yeast-based processes ranging from production of yeast extracts to the expression of heterologous proteins (Kazemi Seresht *et al.*, 2013). The results presented in this study identify transaminase genes as highly interesting targets for empirical optimization of the production of amino acid-derived products by *S. cerevisiae*. However, knowledge-based optimization of the transaminase network will require systematic, quantitative analysis of enzyme kinetics of the different transaminases and their substrate specificities, as well as mathematical modelling of the transaminase network in *S. cerevisiae*.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Table S1. Medium composition for synthetic genetic arrays analysis (SGA).
Table S2. Fluorescence screening results of the SGA mutant collection.
Table S3. Genes differentially expressed between strains IMY081 (*aro8Δ*) and CEN.PK113-7D grown in chemostat culures

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