# Supplementary Methods

## Supplementary Methods 2: Molecular biology techniques

Genomic DNA from *E.coli* (migula) Castellani and Chalmers (ATCC 47076) or *S. cerevisiae* used for strain construction purposes was isolated using the QIAGEN Blood & Cell Culture Kit with 100/G Genomic-tips (Qiagen, Hilden, Germany) or alternatively for yeast with the YeaStar genomic DNA kit (Zymo Research, Irvine, CA). *E.coli* DNA from a mixed population of *E.coli* XL1-Blue and *E.coli* BL21 used for construction of the test NeoChrs was isolated as described by Postma *et al.* 8. Plasmids were isolated from *E.coli* using the GenElute Plasmid Miniprep Kit (Sigma-Aldrich, St. Louis, MO) or the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer’s instructions.

All PCRs for strain construction purposes were performed with Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific) using either desalted or PAGE purified (in case of ORFs) primers (Sigma-Aldrich). PCR products were verified by separation on 1% (w/v) or 2% (w/v) agarose (TopVision Agarose, Thermo Fisher Scientific) gels in 1x Tris-acetate-EDTA (TAE) buffer (Thermo Fisher Scientific) or 1x Tris-Borate-EDTA (TBE) (Thermo Fisher Scientific) buffer. For size determination GeneRuler DNA Ladder mix (Sigma-Aldrich) or GeneRuler DNA Ladder 50bp (Sigma-Aldrich) were used. For DNA staining 10 μL L-1 SERVA (SERVA Electrophoresis GmbH, Heidelberg, Germany) was added to the agarose gel solution. DNA was purified using either the Zymoclean Gel DNA Recovery kit (Zymo Research), the GenElute PCR Clean-Up kit (Sigma-Aldrich), the GeneJET PCR Purification Kit (Thermo Fisher Scientific) or using AMPure XP beads (Beckman Coulter, Brea, CA) according to the suppliers’ protocols. Purity of DNA was checked using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) and the concentration was measured either by the NanoDrop 2000 (Thermo Fisher Scientific) or by the Qubit dsDNA BR Assay kit (Thermo Fisher Scientific) using the Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA). Gibson assembly used to construct gRNA plasmids and some expression plasmids was performed with the NEBuilder® HiFi DNA Assembly Master Mix (New England Biolabs, Ipswich, MA) in a final volume of 5 µL according to the supplier’s instruction.

Chemical *E.coli* XL1-Blue transformation was performed as described by Inoue *et al.* 16 and correct assembly of plasmids was verified by diagnostic PCR or restriction analysis. *S. cerevisiae* was transformed using the lithium acetate/polyethylene glycol method 17. For diagnostic PCR, DNA was isolated by resuspending some culture in 0.2 M NaOH or by using the method described by Looke *et al.* 18. All diagnostic PCRs were performed using DreamTaq PCR Master Mix (Thermo Fisher Scientific) according to the manufacturer’s instruction. For yeast, single colony isolates were obtained by three consecutive re-streaks on solid selective medium.

## Supplementary Methods 3: Detailed construction of the host strain IMX2770

Before assembly of coding NeoChrs a suitable starting strain was engineered by several rounds of CRISPR/Cas9 gene deletions as described by Mans *et al.* 9 . As parental strain, the SwYG strain IMX589 from Kuijpers *et al.* 6 was used. In this strain the minor paralogs of glycolysis are deleted and the major paralogs are centralized at the *sga1* locus on chromosome IX. From this strain, the *amdSYM* marker located between the major paralogs of glycolysis was deleted using *in vivo* assembly of a pMEL10 gRNA plasmid backbone (amplified with primer 6005, Supplementary Table 16) and a gRNA insert made from annealing primers (11588 & 11589, Suppl. Table S15). The DSB was repaired with a 120 bp repair fragment homologous to the flanking SHRs K and L, made by annealing of complementary primers (11590 & 11591, Supplementary Table 16), resulting in strain IMX1433 and IMX1769 before and after plasmid recycling, respectively.

Subsequently the minor paralogs of the pentose phosphate pathway, *GND2, NQM1, SOL4* and *TKL2*, were deleted by transformation of two gRNA plasmids (pUDR286 & pUDR590, Supplementary Table 8A) and 120 bp repair fragments (Supplementary Table 17) homologous to the 60 bp upstream and downstream of the ORF. The strains were stocked before and after discarding the gRNA plasmids, resulting in respectively IMX2154 and IMX2204

Next as much as possible of the promoter, gene and terminator of the *ura3* and *his3* as well as of the functional *SpHIS5* gene were removed using gRNA plasmids pUDR426 and pUDR546 and repair fragments (Supplementary Tables 8A and 18) obtaining strain IMX2234 after plasmid recycling.

Finally, in the last round of deletion, the *ARO10* gene was removed with gRNA plasmid pUDR406 and a 120 bp repair fragment (Supplementary Tables 8A and 19). Again, the plasmid was removed and the strain was stocked as IMX2270.

## Supplementary Methods 4: MinION long-read sequencing

Average DNA size and integrity were verified with the TapeStation 2200 (Agilent Technologies, Santa Clara, CA). Before sequencing, flow cell quality was assessed by running the MinKNOW platform QC. All samples were sequenced in-house on a MinION (Oxford Nanopore). Samples NeoChr10.10, NeoChr10.13 (IMF22), Neochr10.47, NeoChr10.54, NeoChr10.16, NeoChr10.62, NeoChr10.67, NeoChr10.69, NeoChr11.19, NeoChr11.22, NeoChr25.25, NeoChr25.47, NeoChr25.53, NeoChr25.56 (IMF27), NeoChr26.2, NeoChr26.4 (IMF29), NeoChr26.6, Neochr26.9 and NeoChr26.1 were sequenced on a FLO-MIN106 flowcell with sequencing kit SQK-LSK108.

Samples NeoChr12 (IMF23), NeoChr30 (IMF41), NeoChr31 (IMF42), NeoChr33 (IMF47) and NeoChr34 (IMF48) were sequenced on a FLO-MIN111 with sequencing kit SQK-LSK109. Basecalling was performed for samples with NeoChr10 and Neochr11 by using Albacore (version 2.3.1, Oxford Nanopore). Demultiplexing of the fastq files of the NeoChr10 and NeoChr11 samples was performed with Porechop (<https://github.com/rrwick/Porechop>). Basecalling and demultiplexing was performed with Guppy (Oxford Nanopore) for samples with NeoChr25 and NeoChr26 with version 3.1.5, samples IMF41, IMF42, IMF47 with version 4.4.2 and IMF48 with version 4.5.4. All resulting fastq files were filtered on length (> 1kb) followed by *de novo* assembly by Canu version 2.0 19.

## Supplementary Methods 5: Analysis of aromatics

### HPLC analysis of aromatic compounds up until naringenin

For extracellular aromatic compounds, a sample containing broth was mixed 1:1 with 96% ethanol, vortexed thoroughly, spun down for 5 minutes at 14800 rpm and the supernatant was used for further analysis. The aromatic compounds up until naringenin (2-phenylethanol (2PE), *p-*hydroxyphenylethanol (*p*OH2PE), phenylacetic acid (PAA), *p-*hydroxyphenylacetic acid (*p*OHPAA), phenylpyruvic acid (PPY), coumaric acid (COUM), cinnamic acid (CIN), phloretic acid (PHLOR) and naringenin (NAR) were measured using an Agilent Zorbax Eclipse plus C18 column (4.6 x 100mm, 3.5 μm) (Agilent). As mobile phase, 0.020 M KH2PO4 set at pH 2.0 containing 1% acetonitrile was used at a flow rate of 0.8 mL min-1 at an operating temperature of 40°C. The amount of acetonitrile was gradually increased to 10% within 6 minutes, then to 40% after 23 minutes, followed by a decrease in amount to 1% after 30 minutes. The compounds were detected using a diode array and a multiple wavelength detector (Agilent G1315C) at different wavelengths: 200 nm for PAA, 210 nm for PPY, 214 nm for 2PE, *p*OH2PE, *p*OHPAA and PHLOR, 270 nm for CIN and finally 280 nm for NAR and COUM.

The extracellular concentrations in the supernatant of the aromatic compounds kaempferol (KEA), dihydrokaempferol (DHK), kaempferol 3-O-glucoside (K3G), pelargonidin (PEL) and pelargonidin 3-O-glucoside (P3G) were detected using LS-MS/MS, as described in the next section. Additionally, since P3G has never been measured extracellular before, the intracellular concentrations of P3G, and its precursors kaempferol, dihydrokaempferol, K3G and pelargonidin were also measured. A certain amount of cell culture was spun down for 5 minutes at 5000 rpm, washed once with dH2O, resuspended in 0.5-1 ml methanol (0.75% HCL) and the samples were stored overnight at -80°C. Next, the samples were lyophilized for 24 h using a Mini Lyotrap freeze-dryer (LTE Scientific TLD, UK) operated at -80 °C, connected to a Pirani 501 manometer (Edwards Vacuum, UK) using a RV8 pump (Edwards Vacuum, UK). Finally, the pellet was resuspended in 1 mL methanol (2.0% HCL) and stored overnight at -80 °C.

### Mass spectrometric analysis of anthocyanin pathway compounds

Identification and quantification of compounds from the anthocyanin pathway downstream of naringenin was performed using an ACQUITY UPLC chromatography system (Waters, UK) coupled online to a high-resolution Orbitrap mass spectrometer (Q-Exactive Focus, Thermo Fisher Scientific, Germany). For chromatographic separation, a reverse phase separation column (ACQUITY UPLC BEH C18, 1.0 mm × 100 mm, 3 μm particle size, part No 186002346, Waters UK) was operated at room temperature using H2O plus 0.1% formic as mobile phase A, and acetonitrile plus 0.1% formic acid as mobile phase B. A gradient was maintained at 50 μL/min at 7.5% B over 5 minutes. Solvent B was then increased to 80% over 4 minutes, and kept constant for additional 3 minutes before equilibrating back to the starting conditions. The metabolite extracts were taken from -80°C immediately before injection, brought to room temperature, vortexed and 15 μL crude extract were mixed with 85 μL 1 mM HCl. The mixture was carefully vortexed and centrifuged using a bench top centrifuge for 1 minute to remove insoluble materials. 5 μL were subsequently injected onto the UPLC reverse phase separation system. The mass spectrometer was operated alternating in full scan and PRM mode. Full scan was acquired from 250–700 m/z in ESI positive mode (+ 3.25 kV), at a resolution of 70 K. Parallel reaction monitoring was performed for the precursor masses for dihydrokaempferol (DHK, Cas No. 104486-98-8) 289.07 m/z [M+H]+ using a NCE of 26, kaempferol (KEA, Cas No. 520-18-3) 287.05 m/z [M+H]+ using a NCE of 30, kaempferol 3-O-glucoside (K3G, Cas No. 480-10-4) 449.10 m/z [M+H]+ using a NCE of 24, pelargonidin (PEL, Cas No. 134-04-3) 271.06 m/z [M]+ using a NCE of 30 and pelargonidin 3-O-glucoside (Cas No. 18466-51-8) m/z 433.10 [M]+ using a NCE of 24. Fragment ions were measured at fixed first mass of 75 m/z, a resolution of 35K, a max IT of 100 ms and an AGC target of 1e5, by acquiring 2 microscans. Raw data were analyzed using XCalibur 4.1 (Thermo) where retention and unique fragments for each individual compound were compared to commercial standards. For quantification, peak intensities of identified compounds from the samples were summed using Matlab 2020b, and compared against an external calibration curve established using commercial standards. The standards were purchased from Sigma Aldrich (dihydrokaempferol Cat No. 91216, kaempferol Cat No. 60010, kaempferol 3-O-glucoside Cat No. PHL89237, pelargonidin chloride Cat No. PHL80084, pelargonidin 3-O-glucoside chloride Cat No. PHL89753). The mass spectrometer was calibrated using the Pierce™ LTQ ESI positive ion calibration solution (Thermo Fisher Scientific, Germany).

## References

1. Boonekamp, F.J. et al. Design and experimental evaluation of a minimal, innocuous watermarking strategy to distinguish near-identical DNA and RNA sequences. *ACS Synth. Biol.* **9**, 1361-1375 (2020).

2. Lee, M.E., DeLoache, W.C., Cervantes, B. & Dueber, J.E. A highly characterized yeast toolkit for modular, multipart assembly. *ACS Synth. Biol.* **4**, 975-986 (2015).

3. Keren, L. et al. Promoters maintain their relative activity levels under different growth conditions. *Mol. Syst. Biol.* **9**, 701 (2013).

4. Knijnenburg, T.A. et al. Combinatorial effects of environmental parameters on transcriptional regulation in *Saccharomyces cerevisiae*: a quantitative analysis of a compendium of chemostat-based transcriptome data. *BMC genomics* **10**, 53 (2009).

5. Boonekamp, F.J. et al. The genetic makeup and expression of the glycolytic and fermentative pathways are highly conserved within the *Saccharomyces* genus. *Front. Genet.* **9**, 504 (2018).

6. Kuijpers, N.G. et al. Pathway swapping: Toward modular engineering of essential cellular processes. *Proc. Natl. Acad. Sci. U.S.A.* **113**, 15060-15065 (2016).

7. Entian, K.-D. & Kötter, P. 25 Yeast genetic strain and plasmid collections in *Methods in Microbiology* (eds. I. Stansfield & M.J.R. Stark) 629-666 (Academic Press, 2007).

8. Postma, E.D. et al. A supernumerary designer chromosome for modular *in vivo* pathway assembly in *Saccharomyces cerevisiae*. *Nucleic Acids Res.* **49**, 1769-1783 (2021).

9. Mans, R. et al. CRISPR/Cas9: a molecular Swiss army knife for simultaneous introduction of multiple genetic modifications in *Saccharomyces cerevisiae*. *FEMS Yeast Res.* **15**, 1-15 (2015).

10. Hassing, E.J., de Groot, P.A., Marquenie, V.R., Pronk, J.T. & Daran, J.G. Connecting central carbon and aromatic amino acid metabolisms to improve *de novo* 2-phenylethanol production in *Saccharomyces cerevisiae*. *Metab. Eng.* **56**, 165-180 (2019).

11. Levisson, M. et al. Engineering *de novo* anthocyanin production in *Saccharomyces cerevisiae*. *Microb. Cell Fact.* **17**, 103 (2018).

12. Koopman, F. et al. *De novo* production of the flavonoid naringenin in engineered *Saccharomyces cerevisiae*. *Microb. Cell Fact.* **11**, 155 (2012).

13. Mitchell, L.A. & Boeke, J.D. Circular permutation of a synthetic eukaryotic chromosome with the telomerator. *Proc. Natl. Acad. Sci. U.S.A.* **111**, 17003-17010 (2014).

14. Verduyn, C., Postma, E., Scheffers, W.A. & Van Dijken, J.P. Effect of benzoic acid on metabolic fluxes in yeasts: A continuous-culture study on the regulation of respiration and alcoholic fermentation. *Yeast* **8**, 501-517 (1992).

15. Luttik, M.A. et al. The *Saccharomyces cerevisiae ICL2* gene encodes a mitochondrial 2-methylisocitrate lyase involved in propionyl-coenzyme A metabolism. *J. Bacteriol.* **182**, 7007-7013 (2000).

16. Inoue, H., Nojima, H. & Okayama, H. High efficiency transformation of *Escherichia coli* with plasmids. *Gene* **96**, 23-28 (1990).

17. Gietz, R.D. & Woods, R.A. Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. *Methods Enzymol.* **350**, 87-96 (2002).

18. Looke, M., Kristjuhan, K. & Kristjuhan, A. Extraction of genomic DNA from yeasts for PCR-based applications. *BioTechniques* **50**, 325-328 (2011).

19. Koren, S. et al. Canu: scalable and accurate long-read assembly via adaptive k-mer weighting and repeat separation. *Genome Res.* **27**, 722-736 (2017).