

Original Research Article

Metabolic engineering of *Saccharomyces cerevisiae* for *de novo* production of dihydrochalcones with known antioxidant, antidiabetic, and sweet tasting properties



Michael Eichenberger^{a,b}, Beata Joanna Lehka^{c,d}, Christophe Folly^a, David Fischer^a, Stefan Martens^e, Ernesto Simón^a, Michael Naesby^{a,*}

^a Evolva SA, Duggingerstrasse 23, 4153 Reinach, Switzerland

^b Department of Biology, Technical University Darmstadt, Schnittspahnstrasse 10, 64287 Darmstadt, Germany

^c Evolva Biotech A/S, Lerso Parkallé 42, 2100 Copenhagen, Denmark

^d Department of Science and Environment, Roskilde University, Universitetsvej 1, 4000 Roskilde, Denmark

^e Department of Food Quality and Nutrition, Fondazione Edmund Mach, Centro Ricerca e Innovazione, Via E. Mach 1, 38010 San Michele all'Adige (TN), Italy

ARTICLE INFO

Chemical compounds studied in this article:

Phloretin (PubChem CID: 4788)

Naringenin (PubChem CID: 932)

Pinoembrin (PubChem CID: 68071)

Phlorizin (PubChem CID: 6072)

Nothofagin (PubChem CID: 42607691)

Trilobatin (PubChem CID: 6451798)

Naringin dihydrochalcone (PubChem CID: 9894584)

3-Hydroxyphloretin (PubChem CID: 11778945)

Keywords:

Dihydrochalcone

Phlorizin

Nothofagin

Naringin dihydrochalcone

Saccharomyces cerevisiae

Double bond reductase

ABSTRACT

Dihydrochalcones are plant secondary metabolites comprising molecules of significant commercial interest as antioxidants, antidiabetics, or sweeteners. To date, their heterologous biosynthesis in microorganisms has been achieved only by precursor feeding or as minor by-products in strains engineered for flavonoid production. Here, the native *ScTSC13* was overexpressed in *Saccharomyces cerevisiae* to increase its side activity in reducing *p*-coumaroyl-CoA to *p*-dihydrocoumaroyl-CoA. *De novo* production of phloretin, the first committed dihydrochalcone, was achieved by co-expression of additional relevant pathway enzymes. Naringenin, a major by-product of the initial pathway, was practically eliminated by using a chalcone synthase from barley with unexpected substrate specificity. By further extension of the pathway from phloretin with decorating enzymes with known specificities for dihydrochalcones, and by exploiting substrate flexibility of enzymes involved in flavonoid biosynthesis, *de novo* production of the antioxidant molecule nothofagin, the antidiabetic molecule phlorizin, the sweet molecule naringin dihydrochalcone, and 3-hydroxyphloretin was achieved.

1. Introduction

Since phlorizin was identified in apples as the first dihydrochalcone (DHC) in 1835 (Petersen, 1835), there has been significant interest in this class of compounds, accompanied by scientific research into their potential benefits for humans. While DHCs have long been thought to be restricted to plants belonging to about 30 plant families (Ninomiya and Koketsu, 2013), DHCs were more recently found in significant

amounts in grapes and raspberries, and with the development of novel and more sensitive analytical methods it is becoming obvious that they might be more widespread (Carvalho et al., 2013; Vrhovsek et al., 2012). Apples however remain unique in that they accumulate DHCs to very high concentrations of up to 14% dry weight in leaves (Gosch et al., 2010b). Although knowledge about the functional role of DHCs *in planta* is limited, many of these structures have received attention for other reasons, mostly relating to a variety of human health and food

Abbreviations: CHI, chalcone isomerase; CHS, chalcone synthase; CH3H, chalcone 3-hydroxylase; CPR, cytochrome P450 reductase; CYP, cytochrome P450; C4H, cinnamate 4-hydroxylase; DBR, double bond reductase; DHC, dihydrochalcone; EMA, European Medicine Agency; FDA, Food and Drug Administration; F3H, flavanone 3 β -hydroxylase; F3'H, flavonoid 3'-hydroxylase; FLS, flavonol synthase; HRT, homologous recombination tag; NDC, naringin dihydrochalcone; NHDC, neohesperidin dihydrochalcone; OD₆₀₀, optical density at 600 nm; OMT, O-methyltransferase; PAL, phenylalanine ammonia lyase; STS, stilbene synthase; UGT, UDP-dependent-glycosyltransferase; VLC, very long chain; 4CL, 4-coumarate-CoA ligase

* Corresponding author.

E-mail address: michaeln@evolva.com (M. Naesby).

<http://dx.doi.org/10.1016/j.jymben.2016.10.019>

Received 9 May 2016; Received in revised form 1 September 2016; Accepted 25 October 2016

Available online 31 October 2016

1096-7176/© 2016 Evolva, Duggingerstrasse 23, 4153 Reinach, Switzerland. Published by Elsevier Inc. on behalf of International Metabolic Engineering Society.

This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

applications (Rozmer and Perjési, 2016). Three of the most prominent examples of active DHCs described in literature are briefly outlined here. Phlorizin was found to be a hypoglycemic agent, acting by inhibiting SGLT1 and SGLT2, the human glucose transporters involved in intestinal glucose absorption and renal glucose reabsorption (Ehrenkranz et al., 2005). It was later used as a blueprint for the development of over ten synthetic antidiabetic drugs (Chao, 2014), three of which have been approved by the Food and Drug Administration (FDA) and European Medicine Agency (EMA) (Scheen, 2015). Aspalathin and nothofagin, which are naturally found in rooibos (*Aspalathus linearis*), exhibit strong antioxidant activity (Snijman et al., 2009) and are absorbed by the human body as intact glycosides due to the metabolic stability of their C-C-glycosidic bond (Breiter et al., 2011). Naringin dihydrochalcone (NDC) and neohesperidin dihydrochalcone (NHDC), which can be chemically synthesized from citrus flavanones, are sweeteners with one and twenty times the sweetness of saccharin on a molar basis, respectively (Horowitz and Gentili, 1969) with NHDC approved as the food additive E959 in Europe (Janvier et al., 2015).

Chemically, the DHCs comprise a 1,3-diphenylpropan-1-one skeleton. They are further functionalized, mainly on the two aromatic rings, by hydroxylation, methylation, prenylation, glycosylation, and/or polymerization. Just over 200 structurally different DHCs have been identified to date (Rozmer and Perjési, 2016). Their proposed biosynthesis in plants is depicted in Fig. 1. The early pathway from phenylalanine to *p*-coumaroyl-CoA is catalysed by phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumarate-CoA ligase (4CL). These steps are shared with the biosynthesis of

lignans and other polyketides, such as flavonoids or stilbenoids, and have been thoroughly studied and characterized (Gosch et al., 2010b). The action of a double bond reductase (DBR) to form *p*-dihydrocoumaroyl-CoA from *p*-coumaroyl-CoA appears to be required to initiate the biosynthesis of DHCs (Dare et al., 2013a; Gosch et al., 2009). Three different enzymes from apple (*Malus x domestica*) have recently been suggested to catalyse this reaction *in planta* (Dare et al., 2013a; Ibdah et al., 2014). Phloretin, the first committed DHC, is then formed by decarboxylative condensation with three units of malonyl-CoA and a subsequent cyclisation, all catalysed by chalcone synthase (CHS). CHS was found to be shared between the flavonoid and DHC pathways in apple (Gosch et al., 2009). Except for a few UDP-dependent-glycosyltransferases (UGTs) from apple, pear, and carnation which can glycosylate the 2'-hydroxygroup of phloretin to form phlorizin (Gosch et al., 2010a; Jugdé et al., 2008; Werner and Morgan, 2009), and two UGTs from rice and buckwheat which are able to C-glycosylate the 3'-position to form nothofagin (Brazier-Hicks et al., 2009; Ito et al., 2014), plant enzymes for further decoration of DHCs still remain to be identified (Fig. 1B). During the preparation of this manuscript, a study was published, reporting that chalcone 3-hydroxylase (CH3H) from *Cosmos sulphureus* can hydroxylate phloretin to form 3-hydroxyphloretin in yeast and in plants (Hutabarat et al., 2016). During the revision of this manuscript, an additional study was published, reporting that *MdPh-4'-OGT* from *Malus x domestica* can glycosylate the 4'-hydroxygroup of phloretin to form trilobatin (Yahya et al., 2016).

Since the early 2000s, technologies arising from systems and synthetic biology have revolutionized metabolic engineering (Xu et al., 2013). Besides academic studies showing the production of

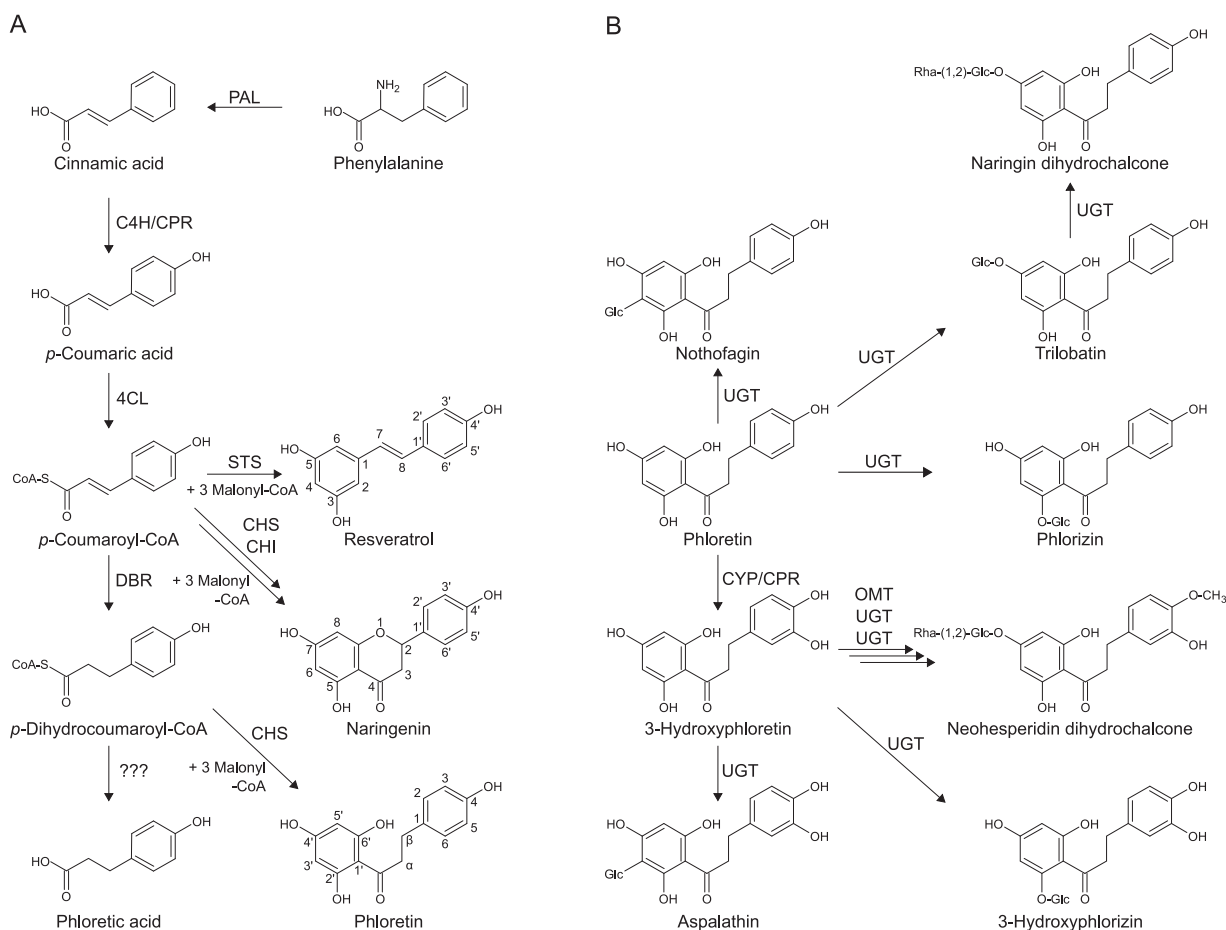


Fig. 1. (A) The biosynthetic pathway to the first committed DHC (phloretin), flavonoid (naringenin), and stilbenoid (resveratrol) from phenylalanine and malonyl-CoA is shown. (B) Depicted are proposed biosynthetic routes to several hydroxylated, glycosylated, and methylated DHC derivatives. CHI, chalcone isomerase; CHS, chalcone synthase; CPR, cytochrome P450 reductase; CYP, cytochrome P450; C4H, cinnamate 4-hydroxylase; DBR, double bond reductase; OMT, O-methyltransferase; PAL, phenylalanine ammonia lyase; STS, stilbene synthase; UGT, UDP-dependent-glycosyltransferase; 4CL, 4-coumarate-CoA ligase.

numerous compounds in microbial hosts, these developments have resulted in commercialization of biological processes for heterologous production of at least 20 products in the fields of bulk chemicals (e.g. 2,4-butanediol, farnesene), pharmaceutical compounds (e.g. artemisinin), fragrances (e.g. nootkatone), food chemicals (e.g. resveratrol, vanillin), and biofuels (e.g. isobutanol, ethanol) (Julleesson et al., 2015). In comparison to extraction from plant materials, such biotechnological processes allow efficient production of specific compounds in an environmentally sustainable way, avoiding the supply chain problems arising from the dependency on plant growth (Krivoruchko and Nielsen, 2015).

Flavonoids and stilbenoids have both been very popular targets in many metabolic engineering publications and two recent reviews give a good overview of the field (Pandey et al., 2016; Wang et al., 2016). On the other hand, DHCs have been the subject of only three studies. Two of these studies (Watts et al., 2004; Werner et al., 2010) expressed 4CL and CHS enzymes in *Escherichia coli* and *Saccharomyces cerevisiae*, respectively, and produced phloretin by feeding phloretic acid. This strategy circumvents the critical DBR step needed to generate the starter molecule for DHC biosynthesis. In another study, a CHI and an enoate reductase from *Eubacterium ramulus* was expressed in *E. coli* and the compounds phloretin, 3-hydroxyphloretin, and homoeriodictyol DHC were produced by feeding naringenin, eriodictyol, and homoeriodictyol, respectively (Gall et al., 2014). However, besides the high cost of these precursors, this strategy additionally required anaerobic cultivation to achieve sufficient enzymatic activity.

In this study we demonstrated the utility of *S. cerevisiae* as microbial cell factory by showing, as a proof of concept, the *de novo* production of various DHCs of commercial interest. A side activity of the native ScTsc13 to reduce *p*-coumaroyl-CoA was used for constructing a biosynthetic pathway to phloretin. Naringenin as a major by-product was substantially decreased by using a highly selective barley CHS for the last step of the pathway. The phloretin pathway was then extended to produce several decorated DHCs using previously identified enzymes, some of which were shown to exhibit sufficient substrate flexibility outside their normal functions in flavonoid biosynthesis.

2. Materials and methods

2.1. Chemicals

Unless stated otherwise, chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Authentic standards of cinnamic acid, *p*-coumaric acid, naringenin, NDC, pinocembrin, phloretic acid, and phlorizin were purchased from Sigma-Aldrich. Authentic standards of phloretin and trilobatin were purchased from Extrasynthese (Genay Cedex, France). Authentic standards of 3-hydroxyphloretin and pinocembrin DHC were purchased from PlantMetaChem (Giessen, Germany) and AnalytiCon (Potsdam, Germany), respectively. Nothofagin was kindly provided by Dr. Alexander Gutmann (Graz University, Austria).

2.2. Strains, plasmids, and media

E. coli XL10 Gold (Agilent, Santa Clara, California, USA) cells were used for subcloning of genes. Table 1 lists all plasmids constructed in this work. Coding sequences for selected enzymes were cloned into expression cassettes of plasmids pEVE2176 to pEVE2181 for assembly by *in vivo* homologous recombination (see Section 2.4). Generally, cloning was done by restriction enzyme and ligation based cloning with *HindIII* HF, *SacII*, and T4 DNA ligase (all New England Biolabs, Ipswich, Massachusetts, USA) according to standard protocols (Green and Sambrook, 2012). Genes containing internal *HindIII* or *SacII* sites were cloned by In-Fusion (Takara, Kyoto, Japan) according to the manufacturer's instructions. *E. coli* was grown in LB medium prepared with 25 g/l of LB broth (Miller) and supplemented with 100 µg/l

ampicillin for amplification of plasmids.

S. cerevisiae strain BG (*MATα hoΔ0 his3Δ0 leu2Δ0 ura3Δ0 cat5Δ0::CAT5(191M) mip1Δ0::MIP1(A661T) gal2Δ0::GAL2 sal1Δ0::SAL1*), which is a derivative of the S288C strain NCYC 3608 (NCYC, Norwich, United Kingdom), was used for all experiments in this work. The strain had been further modified in our labs. Briefly, the *LEU2* and *HIS3* open reading frames were deleted to create two additional auxotrophies for leucine and histidine, respectively. The *KanMX* cassette was excised by Cre-Lox recombination. The non-functional *gal2* gene was replaced with a functional allele from *S. cerevisiae* SK1 strain NCYC 3615 (NCYC) and the *sal1*, *mip1*, and *cat5* genes were engineered to reduce petite formation (Dimitrov et al., 2009). Yeast cultures were grown in SC medium prepared with 1.47 g/l Synthetic Complete (Kaiser) Drop Out: Leu, His, Ura (Formedium, Hunstanton, United Kingdom), 6.7 g/l Yeast Nitrogen Base Without Amino Acids, 20 g/l D-(+)-Glucose, pH set to 5.8 with hydrochloric acid, and supplemented with 76 mg/l histidine, 380 mg/l leucine, and/or 76 mg/l uracil depending on the plasmids to be maintained in the strains.

2.3. Genes

Table 1 shows all genes used in this study, as well as the vector backbones they were cloned into. Coding sequences of all genes can be found in Supplementary Table 4. *S. cerevisiae* codon optimized genes were manufactured by GeneArt (ThermoFisher, Waltham, Massachusetts, USA), except genes in pDHC25 and pDHC26, which were obtained by site directed mutagenesis of pDHC24 using overlap extension PCR with primers DHPR1 to DHPR10 (Supplementary Table 1) according to a standard protocol (Heckman and Pease, 2007). During synthesis or PCR, all genes were provided at the 5' end with an AAGCTTAAA DNA sequence comprising a *HindIII* site and a Kozak sequence and at the 3' end a GGCGCC DNA sequence comprising a *SacII* restriction site. Genes in pDHC3, pDHC6, and pDHC7 were amplified by PCR (Q5 DNA polymerase, New England Biolabs) with primers DHPR11 to DHPR16 (Supplementary Table 1) from a colony of *S. cerevisiae* S288C after lysis in 30 µl 0.2% SDS at 95 °C for 5 min and clarification at 14,000g for 5 min. For the genes in pDHC7 and pDHC8, restriction sites and Kozak sequence, as described above, were added during PCR. *ScCPR1* in pDHC3 was initially cloned with *SpeI* and *XhoI*, because it contained an internal *SacII* site. This site was then removed with a silent mutation (c519t) introduced by inverse PCR using primers DHPR17 and DHPR18 according to standard protocols (Green and Sambrook, 2012). For genes in pDHC4, pDHC22, pDHC23, pDHC30, pDHC31, pDHC33, pDHC38, and pDHC39, RNA was extracted from *Arabidopsis thaliana* leaves or *M. x domestica* var. Golden Delicious apple peel with the RNeasy kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. First strand cDNA was synthesized with the Mint-2 cDNA synthesis kit (Evrogen, Moscow, Russia) and genes were amplified by PCR (Q5 DNA polymerase) with primers DHPR19 to DHPR34 (Supplementary Table 1). During PCR, an AAA Kozak sequence was added to the 5' end of the gene and 15 base pairs with homology to the plasmid backbone were added at the 5' and 3' end of the gene for cloning by In-Fusion into plasmid backbones linearized with *HindIII* and *SacII*.

2.4. Assembly of multi-expression plasmids by homologous recombination tags (HRTs)

Multiple expression cassettes were assembled into multi-expression plasmids with an *in vivo* homologous recombination technique slightly modified from DNA assembler (Shao et al., 2009). In contrast to the original version, the 60 base pair homology sequences used to assemble different cassettes were not added by PCR but were already present in HRT entry vectors. This allows indefinite reuse of cassettes after initial

Table 1

List of genes used in this work and HRT backbones they were cloned into.

Plasmid name	Gene	Backbone	Codon optimized	Organism	NCBI protein accession
pDHC1	<i>AtPAL2</i>	pEVE2179	yes	<i>Arabidopsis thaliana</i>	NP_190894.1
pDHC2	<i>AmC4H</i>	pEVE2180	yes	<i>Ammi majus</i>	AAO62904.1
pDHC3	<i>ScCPR1</i>	pEVE2181	no	<i>Saccharomyces cerevisiae</i>	NP_011908.1
pDHC4	<i>At4CL2</i>	pEVE2178	no	<i>Arabidopsis thaliana</i>	NP_188761.1
pDHC5	<i>HaCHS</i>	pEVE2176	yes	<i>Hypericum androsaemum</i>	Q9FUB7.1
pDHC6	<i>ScDFG10</i>	pEVE2177	no	<i>Saccharomyces cerevisiae</i>	NP_012215.1
pDHC7	<i>ScTSC13</i>	pEVE2177	no	<i>Saccharomyces cerevisiae</i>	NP_010269.1
pDHC8	<i>KITSC13</i>	pEVE2177	yes	<i>Kluyveromyces lactis</i>	XP_452392.1
pDHC9	<i>AtECR</i>	pEVE2177	yes	<i>Arabidopsis thaliana</i>	NP_191096.1
pDHC10	<i>GhECR2</i>	pEVE2177	yes	<i>Gossypium hirsutum</i>	NP_001314306.1
pDHC11	<i>MdECR</i>	pEVE2177	yes	<i>Malus x domestica</i>	XP_008382818.1
pDHC12	<i>MdENRL3</i>	pEVE2177	yes	<i>Malus x domestica</i>	NP_001280847.1
pDHC13	<i>MdENRL5</i>	pEVE2177	yes	<i>Malus x domestica</i>	NP_001281005.1
pDHC14	<i>MdHCDBR</i>	pEVE2177	yes	<i>Malus x domestica</i>	XP_008367739.1
pDHC15	<i>ErERED</i>	pEVE2177	yes	<i>Eubacterium ramulus</i>	AGS82961.1
pDHC16	<i>RiZS1</i>	pEVE2177	yes	<i>Rubus idaeus</i>	AEL78825.1
pDHC17	<i>PcCHS</i>	pEVE2176	yes	<i>Petroselinum crispum</i>	P16107.1
pDHC18	<i>PhCHS</i>	pEVE2176	yes	<i>Petunia hybrida</i>	CAA32731.1
pDHC19	<i>HvCHS1</i>	pEVE2176	yes	<i>Hordeum vulgare</i>	P26018.1
pDHC20	<i>HvCHS2</i>	pEVE2176	yes	<i>Hordeum vulgare</i>	Q96562.1
pDHC21	<i>SbCHS</i>	pEVE2176	yes	<i>Scutellaria baicalensis</i>	BAB03471.1
pDHC22	<i>MdCHS1</i>	pEVE2176	no	<i>Malus x domestica</i>	NP_001306186.1
pDHC23	<i>MdCHS2</i>	pEVE2176	no	<i>Malus x domestica</i>	NP_001306181.1
pDHC24	<i>MdUGT88F1</i>	pEVE2176	yes	<i>Malus x domestica</i>	D3UAG5.1
pDHC25	<i>MdUGT88A1</i>	pEVE2176	yes	<i>Malus x domestica</i>	B3TKC8.1
pDHC26	<i>PcUGT88F2</i>	pEVE2176	yes	<i>Pyrus communis</i>	D3UAG3.1
pDHC27	<i>DeGT4</i>	pEVE2176	yes	<i>Dianthus caryophyllus</i>	BAD52006.1
pDHC28	<i>OsCGT</i>	pEVE2176	yes	<i>Oryza sativa</i> subsp. <i>japonica</i>	C3W7B0.1
pDHC29	<i>AtUGT73B2</i>	pEVE2178	yes	<i>Arabidopsis thaliana</i>	NP_567954.1
pDHC30	<i>AtUGT76D1</i>	pEVE2178	no	<i>Arabidopsis thaliana</i>	NP_180216.1
pDHC31	<i>AtUGT84B1</i>	pEVE2178	no	<i>Arabidopsis thaliana</i>	NP_179907.1
pDHC32	<i>Cml_2RHAT</i>	pEVE2179	yes	<i>Citrus maxima</i>	Q8GVE3.2
pDHC33	<i>AtRHM2</i>	pEVE2180	no	<i>Arabidopsis thaliana</i>	NP_564633.2
pDHC34	<i>OsF3'H</i>	pEVE2177	yes	<i>Oryza sativa</i> subsp. <i>japonica</i>	XP_015613041.1
pDHC35	<i>PhF3'H</i>	pEVE2177	yes	<i>Petunia hybrida</i>	Q9SBQ9.1
pDHC36	<i>PfF3'H</i>	pEVE2177	yes	<i>Perilla frutescens</i> var. <i>crispa</i>	BAB59005.1
pDHC37	<i>AcF3'H</i>	pEVE2177	yes	<i>Allium cepa</i>	AAS48419.1
pDHC38	<i>MdF3'H1</i>	pEVE2177	no	<i>Malus x domestica</i>	ACR14867.1
pDHC39	<i>MdF3'H2</i>	pEVE2177	no	<i>Malus x domestica</i>	XP_008374610.1
pDHC40	<i>CsCH3H</i>	pEVE2177	yes	<i>Cosmos sulphureus</i>	ACO35755.1
pDHC41	<i>AtATRI</i>	pEVE2178	yes	<i>Arabidopsis thaliana</i>	NP_194183.1
pDHC42	<i>CaER</i>	pEVE2177	yes	<i>Clostridium acetobutylicum</i>	WP_010966642.1

cloning and sequence verification of the respective genes. After cloning into these entry vectors, both expression cassettes and helper fragments required for assembly of HRT plasmids are flanked by 60 base pair HRTs named A, B, C, D, E, F, G, H, and Z, which are in turn flanked by *AscI* restriction sites for release of the tagged fragments. [Supplementary Table 2](#) lists all basic parts used in this work. Genes of interest were cloned into pEVE2176–2181 as shown in [Table 1](#). For multi-expression HRT plasmid assembly 310 fmol each of the ZA and AB entry plasmids, containing all elements required for replication, maintenance, and selection, were combined with 460 fmol each of all other entry plasmids, containing the expression cassettes and the closing linker. The complete mixture was digested with *AscI* (New England Biolabs) in a 10 μ l reaction, releasing all inserts from their vector backbone, followed by heat inactivation of the restriction enzyme at 80 $^{\circ}$ C for 20 min. The whole reaction was used to transform *S. cerevisiae* with a standard LiAc transformation ([Gietz and Schiestl, 2007](#)). [Supplementary Table 3](#) lists all strains constructed in this work.

2.5. Yeast growth and broth extraction

Yeast batch cultures for production of metabolites were performed with the System Duetz (EnzyScreen, Heemstede, Netherlands). All incubation steps for growth and extraction were performed in an ISF-1-W shaker (Kuhner, Birsfelden, Switzerland) at 30 $^{\circ}$ C, 300 RPM, and 5 cm shaking diameter. Precultures were inoculated in a square 96-

half-deepwell microplate from a single colony in three replicates in 300 μ l SC dropout medium, as required for plasmid selection, and incubated for 24 h. Optical density at 600 nm (OD_{600}) of a 1:50 dilution was measured in an Ultrospec 10 table top spectrophotometer (GE Healthcare, Little Chalfont, United Kingdom). Main cultures were inoculated in 2 ml of the same SC medium in a 24-deepwell microplate to an OD_{600} of 0.1 and incubated for 72 h. Final OD_{600} of a 1:50 dilution was measured and 300 μ l broth was extracted with 300 μ l methanol by incubating for 10 min in a 96 square deepwell microplate and finally clarified by centrifugation at 4000g for 5 min.

2.6. Quantification of compounds by UPLC-MS

Clarified broth extracts were diluted four times with 50% methanol and 2 μ l was injected on a Waters Acquity ultra performance liquid chromatography system coupled to a Waters Acquity triple quadrupole mass detector (Milford, Massachusetts, USA). Separation of the compounds was achieved on a Waters Acquity UPLC[®] BEH C18 column (1.7 μ m, 2.1 mm \times 50 mm) kept at 55 $^{\circ}$ C. Mobile phases A and B were water containing 0.1% formic acid and acetonitrile containing 0.1% formic acid, respectively. A flow of 0.6 ml/min was used. The gradient profile was as follow: 0.3 min constant at 10% B, a linear gradient from 10% B to 25% B in 3.7 min, a second linear gradient from 25% B to 100% B in 1 min, a wash for 1 min at 100% B, and back to the initial condition of 10% B for 0.6 min. The mass

analyzer was equipped with an electrospray source and operated in negative mode. Capillary voltage was 3.0 kV; the source was kept at 150 °C and the desolvation temperature was 350 °C. Desolvation and cone gas flow were 500 l/h and 50 l/h, respectively. $[M-H]^-$ ions of compounds of interest were tracked in SIR mode. Compounds were quantified using a quadratic calibration curve with authentic standards ranging from 0.156 mg/l to 80 mg/l for *p*-coumaric acid and phloretic acid or from 0.039 mg/l to 20 mg/l for all other compounds.

3. Results and discussion

3.1. Testing DBRs for production of phloretin in metabolically engineered yeast

Several previous studies focusing on heterologous production of flavonoids or stilbenoids in *S. cerevisiae* reported the appearance of phloretic acid or phloretin as by-products and attributed this to an unspecific activity of some native yeast DBR acting on *p*-coumarate or *p*-coumaroyl-CoA to form phloretic acid or *p*-dihydrocoumaroyl-CoA, respectively (Beekwilder et al., 2006; Jiang et al., 2005; Koopman et al., 2012; Luque et al., 2014). By screening of 26 homozygous and heterozygous deletion mutants of *S. cerevisiae* for genes that might be involved in this DBR shunt activity, *ScDFG10* and *ScTSC13* were identified as possible candidates (Lehka et al., submitted). *ScDfg10* is a polyprenol reductase involved in the biosynthesis of dolichol, the precursor for protein N-glycosylation (Cantagrel et al., 2010). *ScTsc13* is a very long chain (VLC) enoyl-CoA reductase, catalysing the double bond reduction in each cycle of VLC fatty acid elongation (Kohlwein et al., 2001). These two enzymes were chosen for over-expression in the attempt to construct a pathway to phloretin in *S. cerevisiae*. In addition to the DBRs, the phloretin pathway comprised the genes *AtPAL2*, *AmC4H*, *ScCPR1*, *At4CL2*, and *HaCHS*, which were previously found to encode a functional naringenin pathway in yeast (data not shown). Despite the absence of a chalcone isomerase (CHI), naringenin production was expected in these strains, since the isomerization of naringenin chalcone to naringenin is known to happen spontaneously (Mol et al., 1985). This spontaneous isomerization was also observed for authentic standards of naringenin chalcone, so naringenin was used for indirect quantification of CHS products derived from *p*-coumaroyl-CoA. Preliminary data suggested that over-expression of *ScTSC13* resulted in increased phloretin production without affecting final cell density (Supplementary Fig. S1a). Moreover, essentially no effect was observed on the long chain and VLC free fatty acid profile (Supplementary Figs. S1b and S1c). This suggested that overexpression of *ScTSC13* alone has no impact on the activity of the four-enzyme VLC fatty acid elongation machinery, which additionally comprises *ScELO2* or *ScELO3*, *ScIFA38*, and *ScPHS1* (Dickson, 2008). Obviously, the DBR activity in the phloretin pathway is not the native function of *ScTsc13* and we wondered to what extent this was a unique feature of this enzyme. We therefore tested four homologous enzymes for this activity: *KITsc13* from *Kluyveromyces*

lactis was chosen as an example of a functionally similar enzyme from another yeast. *AtEcr* from *A. thaliana* and *GhEcr2* from cotton were selected as they have previously been shown to complement *S. cerevisiae* lacking *ScTsc13* activity (Gable et al., 2004; Song et al., 2009), thus confirming that they can be functionally expressed in yeast. Additionally, the *M. x domestica* *MdEcr* homologue was selected due to the high DHC content of this plant. On top of that, several recently identified DBRs were tested for activity in our synthetic phloretin pathway: three enzymes from apple were previously shown to be implicated in DHC production, either by RNAi experiments (*MdEnr13*) or by *in vitro* reaction studies (*MdEnr15*, *MdHcdbr*) (Dare et al., 2013a; Ibdah et al., 2014). *ErEred* was selected because it has been shown to produce phloretin from naringenin chalcone when *ErERED* is expressed in anaerobically grown *E. coli* (Gall et al., 2014). Finally, *RiZs1* from raspberry was chosen because it reduces the double bond of 4-hydroxybenzalacetone, which is structurally very similar to *p*-coumaric acid (Koeduka et al., 2011). Twelve HRT plasmids were constructed, co-expressing *AtPAL2*, *AmC4H*, *ScCPR1*, *At4CL2*, and *HaCHS* together with either one of the eleven DBRs or a negative control without DBR, to make the strains DBR1 to DBR12, and production of naringenin and phloretin was quantified (Fig. 2).

Of the DBRs tested, only *ScTsc13* and its close homologue *KITsc13* had any impact on the ratio of phloretin to naringenin. Strain DBR2, overexpressing *ScTSC13*, was most efficient in producing phloretin, reaching a final titer of 42.7 ± 0.9 mg/l and only 13.0 ± 0.6 mg/l of naringenin. As mentioned above, *ScTSC13* is an essential gene in *S. cerevisiae* and the corresponding enzyme catalyses the double bond reduction of VLC trans-2-enoyl-CoA (Kohlwein et al., 2001). Like the native substrates of this enzyme, *p*-coumaroyl-CoA comprises an α,β -unsaturated CoA thioester functional group. Therefore it seems likely that *p*-coumaroyl-CoA is the actual substrate of the *ScTsc13* side activity.

During preparation of this manuscript, it was reported that a 2-enoate reductase, *CaEr*, from *Clostridium acetobutylicum* was able to reduce the double bond of cinnamic acid and *p*-coumaric acid when expressed in *E. coli* (Sun et al., 2016). A phloretin pathway with this DBR was therefore constructed on an HRT plasmid to create strain DBR13 and production of phloretin and naringenin was compared to strains DBR12 (control) and DBR2, overexpressing *ScTSC13*. The ratio of phloretin to naringenin in DBR13 was in the same range as for the control strain, indicating that *CaEr* is not active in the phloretin pathway in yeast (Supplementary Fig. S2).

3.2. Characterization of the phloretin pathway in vivo

The overexpression of *ScTSC13*, together with a heterologous pathway to naringenin chalcone, resulted in production of DHCs instead of flavonoids. In order to further elucidate the exact substrate of *ScTsc13* in this pathway, and to identify potential bottlenecks, six truncated pathways were assembled on HRT plasmids to make strains PAR1–PAR6 (Table 2). These strains were tested for production of

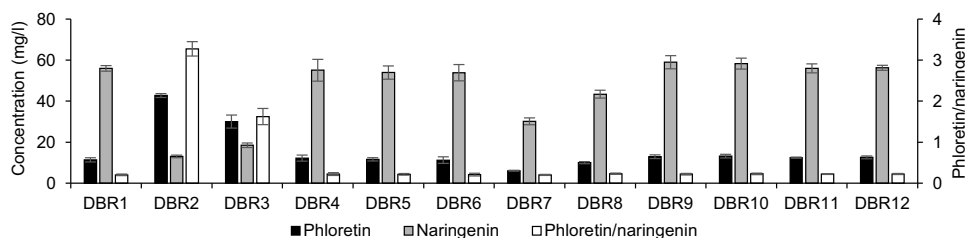


Fig. 2. Production of phloretin (black) and naringenin (grey) and the ratio of produced phloretin to naringenin (white) by strains DBR1 to DBR12, expressing a phloretin pathway with *AtPAL2*, *AmC4H*, *ScCPR1*, *At4CL2*, and *HaCHS* and different DBRs (DBR1: *ScDFG10*; DBR2: *ScTSC13*; DBR3: *KITSC13*; DBR4: *AtEcr*; DBR5: *GhEcr2*; DBR6: *MdEcr*; DBR7: *MdENR13*; DBR8: *MdENR15*; DBR9: *MdHCDR*; DBR10: *ErERED*; DBR11: *RiZS1*; DBR12: no DBR) on an HRT plasmid. Represented are average and standard deviation of three independent cultures.

Table 2

Genes on HRT plasmids in strains PAR1 to PAR6.

Strain	Genes on corresponding HRT plasmid
PAR1	<i>AtPAL2, AmC4H, ScCPR1, At4CL2, HaCHS, ScTSC13</i>
PAR2	<i>AtPAL2, AmC4H, ScCPR1, At4CL2, HaCHS</i>
PAR3	<i>AtPAL2, AmC4H, ScCPR1, At4CL2, ScTSC13</i>
PAR4	<i>AtPAL2, AmC4H, ScCPR1, At4CL2</i>
PAR5	<i>AtPAL2, AmC4H, ScCPR1, ScTSC13</i>
PAR6	<i>AtPAL2, AmC4H, ScCPR1</i>

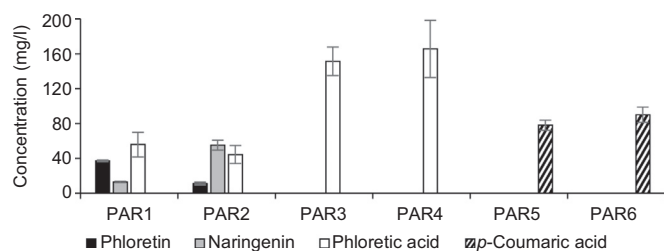


Fig. 3. Production of phloretin (black), naringenin (grey), phloretic acid (white), and *p*-coumaric acid (black hatching) by strains PAR1 to PAR6, containing an HRT plasmid with partial and full phloretin pathways as shown in Table 2. Represented are average and standard deviation of three independent cultures.

cinnamic acid, *p*-coumaric acid, phloretic acid, naringenin, and phloretin (Fig. 3). When the pathway was interrupted at the stage of *p*-coumaric acid by leaving out *At4CL2* and *HaCHS* (strains PAR5 and PAR6) only *p*-coumaric acid was produced. No phloretic or cinnamic acid was detected despite the overexpression of *ScTSC13*. This suggested that *AmC4h* was not limiting in the pathway and that *p*-coumaric acid was not a substrate of *ScTsc13*. When the pathway was extended to include *At4CL2* (strains PAR3 and PAR4) the yeast produced only phloretic acid, whereas the *p*-coumaric acid concentration was below the detection limit. This suggested that *At4cl2* efficiently activated *p*-coumaric acid to *p*-coumaroyl-CoA, which was then reduced to *p*-dihydrocoumaroyl-CoA, even without overexpression of *ScTSC13*. *p*-Dihydrocoumaroyl-CoA was subsequently hydrolysed to phloretic acid, by an unspecific enzymatic reaction or spontaneously. These results also showed that *At4cl2* and *ScTsc13* were not limiting under these experimental conditions.

Strains PAR1 and PAR2, comprising the full length pathway to naringenin chalcone, both produced phloretin and naringenin. The accumulation of both compounds most likely reflected the competition between *ScTsc13* and *HaChs* for *p*-coumaroyl-CoA. As expected, the overexpression of *ScTSC13* (PAR1) clearly shifted the balance of production towards more phloretin and less naringenin compared to PAR2. Both strains accumulated phloretic acid, suggesting slow conversion of *p*-dihydrocoumaroyl-CoA to phloretin by *HaChs*, which seemed to present a bottleneck in the current pathway. To confirm the gradual build-up of compounds measured after 72 h, we performed a time course experiment using PAR1, analysing the accumulation of relevant compounds (Supplementary Fig. S3). After a minor transient accumulation of *p*-coumaric acid during the initial exponential growth phase on glucose, there was a steady build-up of phloretic acid, as well as phloretin and naringenin, corresponding to a presumed slow reaction of *HaChs*.

These results support the hypothesis that *p*-coumaroyl-CoA is a substrate of *ScTsc13* although we cannot completely exclude the possibility that the enzyme has activity on naringenin chalcone. However, this seems very unlikely, since naringenin chalcone does not share any structural similarity, nor the CoA functional group, with the natural substrates of the enzyme (Kohlwein et al., 2001). Furthermore, naringenin chalcone quickly isomerizes to naringenin, which does not contain the targeted double bond, and therefore is not a substrate of *ScTsc13*. In fact, in the current pathway naringenin is an

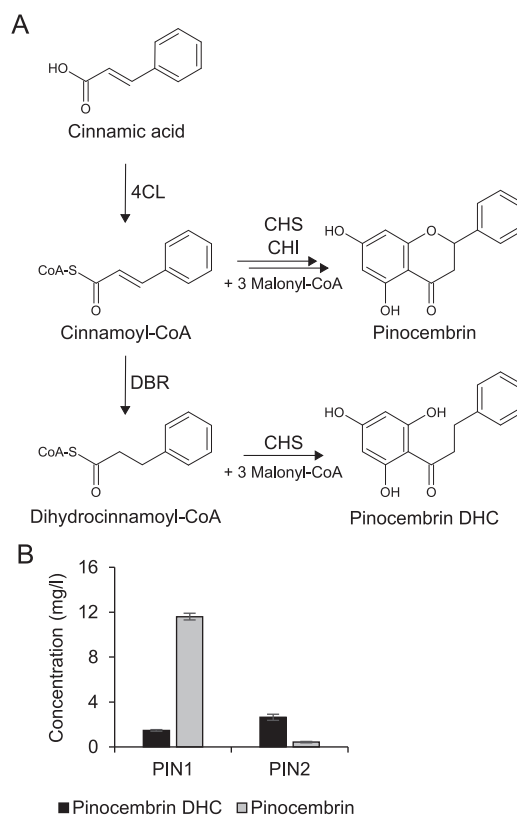


Fig. 4. (A) Biosynthetic route from cinnamic acid to pinocembrin and proposed route to pinocembrin DHC. (B) Production of pinocembrin DHC (black) and pinocembrin (grey) by strains PIN1 and PIN2, expressing a pathway to pinocembrin chalcone with *AtPAL2*, *At4CL2*, and *HaCHS* either without (PIN1) or with (PIN2) overexpression of *ScTSC13*, respectively. Represented are average and standard deviation of three independent cultures. CHI, chalcone isomerase; CHS, chalcone synthase; DBR, double bond reductase; 4CL, 4-coumarate-CoA ligase.

unwanted by-product and, hence, a CHS that does not accept *p*-coumaroyl CoA would be highly desirable. This would greatly simplify balancing of the pathway, during further boosting and optimisation, as there would be no competition with *ScTsc13* for the same substrate. While *HaChs* represents the major limiting step to be alleviated in the heterologous pathway, another way to further increase phloretin production would be to increase the flux into the pathway, e.g. by boosting phenylalanine production and increasing PAL activity. This approach successfully increased production of naringenin (Koopman et al., 2012), *p*-coumaric acid (Rodriguez et al., 2015), and resveratrol (Li et al., 2015) in previous studies, which all share phenylalanine as precursor.

3.3. Using *ScTSC13* for production of pinocembrin DHC

While most DHCs found in nature are derived from phloretin, some plants, such as *Uvaria angolensis* (Hufford and Oguntimein, 1980) or *Mitrella kentia* (Benosman et al., 1997), accumulate also DHC structures lacking the 4-hydroxy group. This means they are most likely derived from pinocembrin DHC and are produced by CHS from dihydrocinnamoyl-CoA and three units of malonyl-CoA. Hence, the biosynthesis of these compounds would require a DBR acting on cinnamoyl-CoA to form dihydrocinnamoyl-CoA (Fig. 4A). In order to test whether *ScTsc13* can also catalyse this reaction, two HRT plasmids were constructed, creating strains PIN1 and PIN2, comprising the pathway to pinocembrin chalcone (*AtPAL2*, *At4CL2*, and *HaCHS*) either with or without additional overexpression of *ScTSC13*. These strains were cultured and the production of pinocembrin and pinocembrin DHC was analysed (Fig. 4B). Strain PIN1, containing only the

native expression of *ScTSC13*, produced 1.47 ± 0.07 mg/l of pinocembrin DHC in addition to 11.6 ± 0.3 mg/l of pinocembrin. Overexpression of *ScTSC13* in strain PIN2 increased the pinocembrin DHC production to 2.6 ± 0.3 mg/l and decreased the pinocembrin production to 0.43 ± 0.06 mg/l, demonstrating that *ScTsc13* also accepts cinnamoyl-CoA. Generally, production of pinocembrin and pinocembrin DHC in strain PIN2 was lower than production of naringenin and phloretin in strain DBR2, which might suggest that *At4cl2* and *HaChs* prefer the hydroxylated substrates, or that the compounds lacking the 4-hydroxy group are more prone to degradation in yeast.

3.4. Testing CHS from various plant species for more specific phloretin production

The production of phloretin in strain DBR2 was unfortunately not very specific with naringenin accumulating as a major by-product to almost a third of the amount of phloretin produced (Fig. 2). In an attempt to decrease this by-product formation, we tested a collection of eight different CHS enzymes from various plants for their ability to produce naringenin or phloretin in yeast. Two of the CHS genes were amplified from the peel of *M. x domestica* var. Golden Delicious, as this plant is known to accumulate high concentrations of DHCs (Dare et al., 2013b; Gosch et al., 2009). Nine HRT plasmids containing the pathway to *p*-dihydrocoumaroyl-CoA (*AtPAL2*, *AmC4H*, *ScCPR1*, *At4CL2*, *ScTSC13*) together with one of the eight CHS, or no CHS as negative control, were constructed to make strains CHS1 to CHS9 and the production of naringenin and phloretin was quantified (Fig. 5). *HaChs*, which was originally chosen for the highest efficiency in producing naringenin in yeast (data not shown), was found to be the most efficient CHS for phloretin production as well. However, most specific for phloretin production was strain CHS5 expressing *HvCHS2*. The phloretin/naringenin ratio of this strain exceeded the ratio of strain CHS1 expressing *HaCHS* by 17.2 ± 1.7 fold. This result was unexpected because barley has not been reported to produce DHCs. In the original publication describing *HvChs2*, an unusual substrate preference for feruloyl-CoA and caffeoyl-CoA over *p*-coumaroyl-CoA and cinnamoyl-CoA was reported, while *p*-dihydrocoumaroyl-CoA was not tested as substrate. Furthermore, it was noted that the sequence of the enzyme was unusually divergent from other CHS sequences (Christensen et al., 1998). Hence, we did a multiple alignment (Supplementary Fig. S4) of the chalcone synthases used in this study and the chalcone synthase from *Medicago sativa*, for which the three dimensional structure was determined (Ferrer et al., 1999). The alignment showed that *HvChs2* contains two amino acid substitutions, T197A and F265I, at otherwise highly conserved residues. According to the predicted structure, the T197A substitution might affect the interaction with the carbonyl group of *p*-coumaroyl-CoA, whereas the F265I would affect the separation of the *p*-coumaroyl-binding pocket from the cyclisation pocket. (Ferrer et al., 1999). Hence, these substitutions might provide some explanation for this unusual preference of *p*-dihydrocoumaroyl-CoA over *p*-coumaroyl-CoA. High substrate specificity may eventually be very

valuable, enabling a more streamlined downstream process, especially in cases like this where product and by-product exhibit a high structural similarity. The lower final phloretin titer achieved in this strain is an issue that most likely can be solved, e.g. by higher copy numbers of *HvCHS2*. A similar strategy of using enzymes as filters for selective production of the compound of interest was recently used in *E. coli* for unnatural C50 carotenoids (Furubayashi et al., 2015).

3.5. Production of the monoglycosylated DHCs phlorizin and nothofagin using known UGTs

In a next step, decorating enzymes were co-expressed in a strain harbouring the phloretin pathway, to evaluate the feasibility and versatility of such a strain as the basic production platform for various DHCs. Strain DBR2 was used for these experiments, not only because it produced more phloretin than strain CHS5, but also because the by-product naringenin could be used as a positive control for functional expression of decorating enzymes with activities on flavanones. First, a set of UGTs with reported activity on DHCs for production of phlorizin (phloretin 2'-*O*-glucoside) (Gosch et al., 2010a; Jugdé et al., 2008; Werner and Morgan, 2009) or nothofagin (phloretin 3'-*C*-glucoside) (Brazier-Hicks et al., 2009) were tested. The corresponding genes were expressed on a second HRT plasmid in strain DBR2, making strains PHZ1 to PHZ4 and strain NOT1. Strain UGTn with an empty second HRT plasmid was used as a negative control. The strains were cultured and production of DHCs was measured. Of the four UGTs reported to glycosylate phloretin to phlorizin, only *MdUgt88a1* and *PcUgt88f2* were efficiently catalysing this reaction in our yeast strain (Fig. 6A). On the other hand, *OsCgt* was highly efficient for nothofagin production, converting phloretin almost completely to this C-glucoside (Fig. 6B).

3.6. Production of NDC using two substrate-promiscuous UGTs

Trilobatin, which is the 4'-*O*-glucoside of phloretin, is a DHC found naturally in *Malus trilobata* and some other *Malus* species (Gosch et al., 2010b; Martens, personal communication). It is an intermediate in a proposed biosynthetic route to the sweetener NDC (Fig. 1B). The equivalent position on the structurally related flavonoids is the 7-hydroxy group (Fig. 1A), and *AtUgt73b2* has previously been described as an enzyme with broad substrate flexibility for different flavonoid backbones (Willits et al., 2004). Two other enzymes, *AtUgt84b1* and *AtUgt76d1*, were shown to be specific for the 7-position of quercetin (Lim et al., 2004). The three genes, encoding these enzymes, were expressed on a second HRT plasmid in strain DBR2, to make strains TRI1 to TRI3, and the production of DHCs was compared to the negative control strain UGTn (Fig. 6C). Only *AtUgt73b2* was able to glycosylate phloretin to trilobatin. Besides producing 33 ± 3 mg/l of trilobatin, it also exhibited minor 2'-*O*-glycosylation activity on phloretin to produce 1.6 ± 0.21 mg/l of phlorizin. The next step in the proposed route to NDC is the rhamnosylation of trilobatin with an α -1,2 glycosidic bond. *Cm1,2Rhat* was reported to catalyse the corresponding reaction on flavanones to form naringenin from naringenin 7-

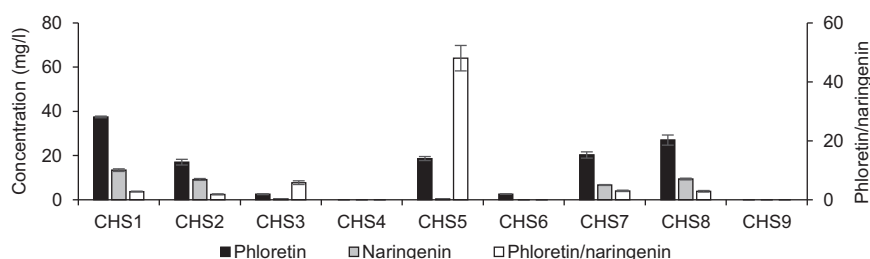


Fig. 5. Production of phloretin (black) and naringenin (grey) and the ratio of produced phloretin to naringenin (white) by strains CHS1 to CHS9, expressing a phloretin pathway with *AtPAL2*, *AmC4H*, *ScCPR1*, *At4CL2*, and *ScTSC13* with different CHS (CHS1: *HaCHS*; CHS2: *PcCHS*; CHS3: *PhCHS*; CHS4: *HvCHS1*; CHS5: *HvCHS2*; CHS6: *SbCHS*; CHS7: *MdCHS1*; CHS8: *MdCHS2*; CHS9: no CHS) on an HRT plasmid. Represented are average and standard deviation of three independent cultures.

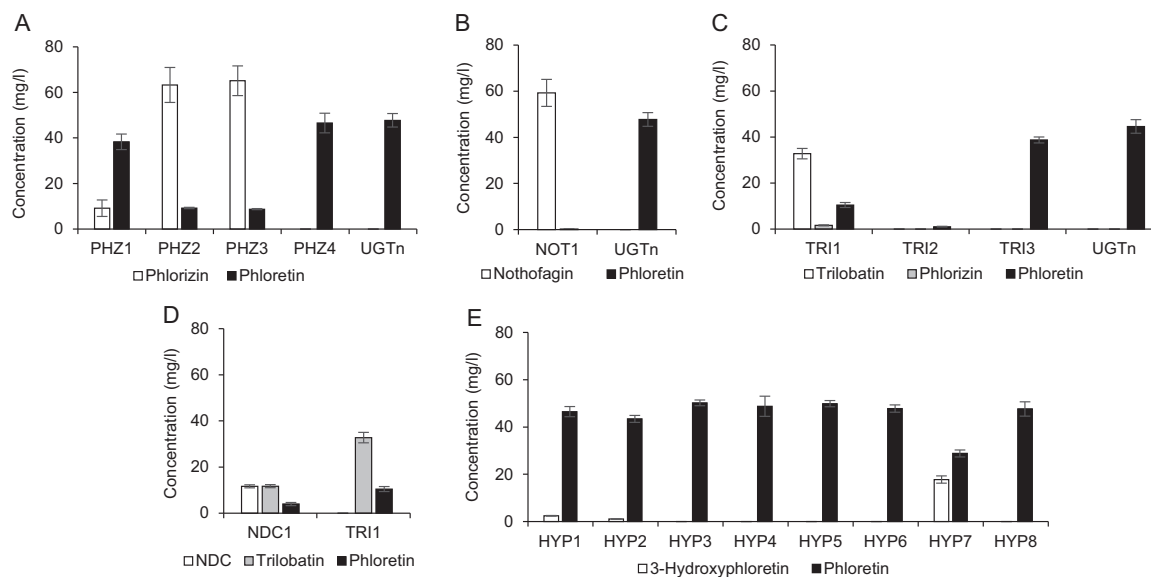


Fig. 6. Engineering of strain DBR2 for production of various phloretin derivatives: (A) Production of phlorizin (white) and phloretin (black) by strains PHZ1 to PHZ4 and UGTn, harbouring a second HRT plasmid, expressing different UGTs or no UGT (PHZ1: *MdUGT88F1*; PHZ2: *MdUGT88A1*; PHZ3: *PcUGT88F2*; PHZ4: *DcGT4*; UGTn: no UGT). (B) Production of nothofagin (white) and phloretin (black) by strains NOT1 and UGTn, harbouring a second HRT plasmid, expressing either *OsCGT* (NOT1) or no enzyme (UGTn). (C) Production of trilobatin (white), phlorizin (grey), and phloretin (black) by strains TRI1 to TRI3 and UGTn, harbouring a second HRT plasmid, expressing different UGTs or no UGT (TRI1: *AtUGT73B2*; TRI2: *AtUGT76D1*; TRI3: *AtUGT84B1*; UGTn: no UGT). (D) Production of NDC (white), trilobatin (grey), and phloretin (black) by strains NDC1 and TRI1, harbouring a second HRT plasmid, expressing either *AtUGT73B2*, *Cm1,2RHAT*, and *AtRHM2* (NDC1) or only *AtUGT73B2* (TRI1). (E) Production of 3-hydroxyphloretin (white) and phloretin (black) by strains HYP1 to HYP8, harbouring a second HRT plasmid, expressing different CYPs or no CYP together with *AtATRI* (HYP1: *OsF3'H*; HYP2: *PhF3'H*; HYP3: *PjF3'H*; HYP4: *AcF3'H*; HYP5: *MdF3'H1*; HYP6: *MdF3'H2*; HYP7: *CsCh3H*; HYP8: no enzyme). Represented are average and standard deviation of three independent cultures.

O-glucoside (Frydman et al., 2004). While UDP-rhamnose, the sugar donor required by *Cm1,2Rhat*, is not naturally produced by *S. cerevisiae*, its biosynthesis has been achieved by expression of *AtRHM2* from *A. thaliana* (Oka et al., 2007). Therefore, we expressed *AtUGT73B2*, *Cm1,2RHAT*, and *AtRHM2* on a second HRT plasmid in strain DBR2 to make strain NDC1. By comparing the production of DHCs in this strain to that of strain TRI3, which expressed only *AtUGT73B2*, we could demonstrate the ability of *Cm1,2Rhat* to accept trilobatin as substrate, allowing the production of 11.6 ± 0.7 mg/l of NDC (Fig. 6D). A similar approach, using promiscuous enzymes, was reported in *E. coli*, where substrate flexibility of flavanone 3 β -hydroxylase (F3H), flavonol synthase (FLS), and flavonoid 3'-hydroxylase (F3'H) was exploited for the assembly of a novel synthetic pathway for the 5-deoxyflavonol fisetin (Stahlhut et al., 2015). Here we adopted this approach, showing that it can be more widely applied across biosynthetic pathways. Interestingly, the enzymes which were eventually selected for constructing the various pathways, all originate from plants that have not been shown to produce DHCs.

3.7. Production of 3-hydroxyphloretin using a substrate promiscuous CYP

Many DHCs, such as 3-hydroxyphlorizin, aspalathin, and neoheperidin DHC, contain a 3-hydroxy group (Fig. 1B). The specific enzymes responsible for the biosynthesis of these compounds have not been identified, although many F3'Hs and one CH3H enzyme have been identified from various plants. These enzymes are regiospecific cytochrome P450s (CYP), which can hydroxylate the equivalent position in the structurally related flavonoids and chalcones. As they are CYPs, they require the action of a cytochrome P450 reductase (CPR) as cofactor. A collection of six F3'H from various plants, plus *CsCh3h*, were tested for their ability to form 3-hydroxyphloretin from phloretin in yeast. Two of the F3'H were amplified from *M. x domestica* var. Golden Delicious cDNA, with the rationale that they might be promiscuous towards DHCs, since apple was reported to contain 3-hydroxyphlorizin (Tsao et al., 2003). Eight HRT constructs containing *AtATRI* together with one of the seven CYPs, or no CYP as negative

control, were assembled in strain DBR2 to make strains HYP1 to HYP8 and production of DHCs was measured (Fig. 6E). While *OsF3'h* and *PhF3'h* showed minor activity on phloretin, *CsCh3h* transformed more than a third of the phloretin to 3-hydroxyphloretin. The apparent inactivity of both apple F3'H proteins is in agreement with previous experience with heterologous expression of F3'Hs from different *Malus* species, known to accumulate 3-hydroxylated DHCs, e.g. *Malus toringo syn sieboldii* (Martens, unpublished). During preparation of this manuscript another study was published, reporting the ability of *CsCh3h* to accept phloretin as substrate, both in yeast and in plants. Overexpression of *CsCh3h* in apple resulted in increased production of 3-hydroxyphlorizin, which correlated with reduced susceptibility to fire blight and scab (Hutabarat et al., 2016).

To summarize, the strain DBR2 was successfully used as a DHC platform strain and the production of various derivatives of phloretin was achieved by using decorating enzymes with known activities on DHC or by exploiting substrate promiscuity of enzymes naturally involved in flavonoid or chalcone biosynthesis. Table 3 gives an overview of the DHCs, for which *de novo* biosynthesis in *S. cerevisiae* was demonstrated in this proof of concept study.

4. Conclusion

DHCs form a class of polyphenols comprising several molecules of considerable commercial interest, e.g. as antioxidants, antidiabetics, and sweeteners. Here we report the *de novo* production of a variety of DHCs in a microbial host by expressing the full length biosynthetic pathways, consisting of between four and nine genes (Table 3).

A critical step for the production of DHCs is the reduction of the α,β -double bond of *p*-coumaroyl-CoA. We tested several previously reported apple reductases, but were not able to show the desired activity with any of these enzymes in yeast. Instead, we took advantage of a side activity of the native yeast VLC enoyl-CoA-reductase *ScTsc13*, normally involved in VLC fatty acid elongation. Upon overexpression, this enzyme efficiently reduced the double bond of *p*-coumaroyl-CoA, thus allowing the production of phloretin. The formation of naringenin, a major by-product of the initial pathway, was then substantially

Table 3

Titers achieved for *de novo* production of different DHCs and numbers of heterologous enzymes overexpressed for the pathways.

Compound	Titer (mg/l)	Enzymes expressed
Phloretin	42.7 ± 0.9	6
Pinocembrin DHC	2.6 ± 0.3	4
Phlorizin	65 ± 7	7
Nothofagin	59 ± 6	7
Trilobatin	32.8 ± 2.3	7
NDC	11.6 ± 0.7	9
3-Hydroxyphloretin	28.8 ± 1.5	8

reduced by using a highly specific barley CHS in the last step of the pathway. Conversely, two non-specific enzymes were employed for assembling the pathways to trilobatin and NDC. We showed that the final glycosylation to these compounds could be performed by UGTs normally involved in the modification of flavonoids.

While the heterologous production of DHCs in this study was reasonably high, a significant increase would be necessary for an economically feasible industrial production process. Two recent studies on metabolic engineering of *p*-coumaric acid (Rodriguez et al., 2015) and resveratrol (Li et al., 2015) in *S. cerevisiae* showed that increasing the pool of aromatic amino acids and cytoplasmic malonyl-CoA, increasing the copy number of rate limiting pathway enzymes, and performing a controlled fed batch fermentation resulted in a substantial increase of product titers, reaching 1.93 g/l for *p*-coumaric acid and 531.41 mg/l for resveratrol. As the precursors of those compounds are shared with DHCs, similar approaches should be applicable for increasing the titers of commercially relevant DHCs.

Competing interests statement

The authors declare competing financial interests in the form of a pending patent application filed by Evolva.

Acknowledgments

This work was supported by the European Union Seventh Framework Programme under Grant agreements no. 613745, Promys and no. 613793, Bachberry. The authors would like to thank Nicholas Milne, Stuart Warner, and Prof. Dr. Beatrix Süss for critical reading of the manuscript and Dr. Alexander Gutmann (Graz University, Austria) for providing an authentic standard of nothofagin.

Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ymben.2016.10.019>.

References

- Beekwilder, J., Wolswinkel, R., Jonker, H., Hall, R., De Vos, C.H., Bovy, A., 2006. Production of resveratrol in recombinant microorganisms. *Appl. Environ. Microbiol.* 72, 5670–5672. <http://dx.doi.org/10.1128/AEM.00609-06>.
- Benosman, A., Oger, J.M., Richomme, P., Bruneton, J., Roussakis, C., Bösch, S., Sévenet, T., Ito, K., Ichino, K., Hadi, A.H.A., 1997. New terpenylated dihydrochalcone derivatives isolated from *Mitrella kentii*. *J. Nat. Prod.* 60, 921–924. <http://dx.doi.org/10.1021/np9700331>.
- Brazier-Hicks, M., Evans, K.M., Gershter, M.C., Puschmann, H., Steel, P.G., Edwards, R., 2009. The C-glycosylation of flavonoids in cereals. *J. Biol. Chem.* 284, 17926–17934. <http://dx.doi.org/10.1074/jbc.M109.009258>.
- Breiter, T., Laue, C., Kressel, G., Gröll, S., Engelhardt, U.H., Hahn, A., 2011. Bioavailability and antioxidant potential of rooibos flavonoids in humans following the consumption of different rooibos formulations. *Food Chem.* 128, 338–347. <http://dx.doi.org/10.1016/j.foodchem.2011.03.029>.
- Cantagrel, V., Lefeber, D.J., Ng, B.G., Guan, Z., Silhavy, J.L., Bielas, S.L., Lehle, L., Hombauer, H., Adamowicz, M., Swiezewska, E., Brouwer, A.P., De, Blui, P., Sykut-Cegielska, J., Houliston, S., Swistun, D., Ali, B.R., Dobyns, W.B., Babovic-Vuksanovic, D., Bokhoven, H., Dobyns, Wevers, R.A., Raetz, C.R.H., Freeze, H.H.,

2010. SRD5A3 is required for converting polyprenol to dolichol and is mutated in a congenital glycosylation disorder. *J. Cell.* 203–217. <http://dx.doi.org/10.1016/j.jcell.2010.06.001>.
- Carvalho, E., Franceschi, P., Feller, A., Palmieri, L., Wehrens, R., Martens, S., 2013. A targeted metabolomics approach to understand differences in flavonoid biosynthesis in red and yellow raspberries. *Plant Physiol. Biochem.* 72, 79–86. <http://dx.doi.org/10.1016/j.plaphy.2013.04.001>.
- Chao, E.C., 2014. SGLT-2 inhibitors: a new mechanism for glycemic control. *Clin. Diabetes* 32, 4–11. <http://dx.doi.org/10.2337/diaclin.32.1.4>.
- Christensen, A.B., Gregersen, P.L., Schröder, J., Collinge, D.B., 1998. A chalcone synthase with an unusual substrate preference is expressed in barley leaves in response to UV light and pathogen attack. *Plant Mol. Biol.* 37, 849–857. <http://dx.doi.org/10.1023/A:1006031822141>.
- Dare, A.P., Tomes, S., Cooney, J.M., Greenwood, D.R., Hellens, R.P., 2013a. The role of enoyl reductase genes in phloridzin biosynthesis in apple. *Plant Physiol. Biochem.* 72, 54–61. <http://dx.doi.org/10.1016/j.plaphy.2013.02.017>.
- Dare, A.P., Tomes, S., Jones, M., McGhie, T.K., Stevenson, D.E., Johnson, R.A., Greenwood, D.R., Hellens, R.P., 2013b. Phenotypic changes associated with RNA interference silencing of chalcone synthase in apple (*Malus domestica*). *Plant J.* 74, 398–410. <http://dx.doi.org/10.1111/tpj.12140>.
- Dickson, R.C., 2008. New insights into sphingolipid metabolism and function in budding yeast. *J. Lipid Res.* 49, 909–921. <http://dx.doi.org/10.1194/jlr.R800003-JLR200>.
- Dimitrov, L.N., Brem, R.B., Kruglyak, L., Gottschling, D.E., 2009. Polymorphisms in multiple genes contribute to the spontaneous mitochondrial genome instability of *Saccharomyces cerevisiae* S288C strains. *Genetics* 183, 365–383. <http://dx.doi.org/10.1534/genetics.109.104497>.
- Ehrenkranz, J.R., Lewis, N.G., Kahn, C.R., Roth, J., 2005. Phlorizin: a review. *Diabetes Metab. Res. Rev.* 21, 31–38. <http://dx.doi.org/10.1002/dmrr.532>.
- Ferrer, J.L., Jez, J.M., Bowman, M.E., Dixon, R. a., Noel, J.P., 1999. Structure of chalcone synthase and the molecular basis of plant polyketide biosynthesis. *Nat. Struct. Biol.* 6, 775–784. <http://dx.doi.org/10.1038/11553>.
- Frydman, A., Weisshaus, O., Bar-Peled, M., Huhman, D.V., Sumner, L.W., Marin, F.R., Lewinsohn, E., Fluhr, R., Gressel, J., Eyal, Y., 2004. Citrus fruit bitter flavors: isolation and functional characterization of the gene Cm1.2RhaT encoding a 1,2 rhamnosyltransferase, a key enzyme in the biosynthesis of the bitter flavonoids of citrus. *Plant J.* 40, 88–100. <http://dx.doi.org/10.1111/j.1365-313X.2004.02193.x>.
- Furubayashi, M., Ikezumi, M., Takaichi, S., Maoka, T., Hemmi, H., Ogawa, T., Saito, K., Tobias, A.V., Umeno, D., 2015. A highly selective biosynthetic pathway to non-natural C50 carotenoids assembled from moderately selective enzymes. *Nat. Commun.* 6, 7534. <http://dx.doi.org/10.1038/ncomms8534>.
- Gable, K., Garton, S., Napier, J.A., Dunn, T.M., 2004. Functional characterization of the *Arabidopsis thaliana* orthologue of Tsc13p, the enoyl reductase of the yeast microsomal fatty acid elongating system. *J. Exp. Bot.* 55, 543–545. <http://dx.doi.org/10.1093/jxb/erh061>.
- Gall, M., Thomsen, M., Peters, C., Pavlidis, I.V., Jonczyk, P., Grünert, P.P., Beutel, S., Scheper, T., Gross, E., Backes, M., Geissler, T., Ley, J.P., Hilmer, J.M., Krammer, G., Palm, G.J., Hinrichs, W., Bornscheuer, U.T., 2014. Enzymatic conversion of flavonoids using bacterial chalcone isomerase and enoate reductase. *Angew. Chem. - Int. Ed.* 53, 1439–1442. <http://dx.doi.org/10.1002/anie.201306952>.
- Gietz, R.D., Schiestl, R.H., 2007. High-efficiency yeast transformation using the LiAc / SS carrier DNA / PEG method. *Nat. Protoc.* 2, 31–34. <http://dx.doi.org/10.1038/nprot.2007.13>.
- Gosch, C., Halbwirth, H., Stich, K., 2010b. Phloridzin: biosynthesis, distribution and physiological relevance in plants. *Phytochemistry* 71, 838–843. <http://dx.doi.org/10.1016/j.phytochem.2010.03.003>.
- Gosch, C., Halbwirth, H., Kuhn, J., Miosic, S., Stich, K., 2009. Biosynthesis of phloridzin in apple (*Malus domestica* Borkh.). *Plant Sci.* 176, 223–231. <http://dx.doi.org/10.1016/j.plantsci.2008.10.011>.
- Gosch, C., Halbwirth, H., Schneider, B., Hölscher, D., Stich, K., 2010a. Cloning and heterologous expression of glycosyltransferases from *Malus x domestica* and *Pyrus communis*, which convert phloretin to phloretin 2'-O-glucoside (phloridzin). *Plant Sci.* 178, 299–306. <http://dx.doi.org/10.1016/j.plantsci.2009.12.009>.
- Green, M.R., Sambrook, J., 2012. *Molecular Cloning: A Laboratory Manual Fourth edition*. Cold Spring Harbor Laboratory Press.
- Heckman, K.L., Pease, L.R., 2007. Gene splicing and mutagenesis by PCR-driven overlap extension. *Nat. Protoc.* 2, 924–932. <http://dx.doi.org/10.1038/nprot.2007.132>.
- Horowitz, R.M., Gentili, B., 1969. Taste and structure in phenolic glycosides. *J. Agric. Food Chem.* 17, 696–700. <http://dx.doi.org/10.1021/jf60164a049>.
- Hufford, C.D., Oguntimein, B.O., 1980. Dihydrochalcones from *Uvaria angolensis*. *Phytochemistry* 19, 2036–2038. [http://dx.doi.org/10.1016/0031-9422\(80\)83036-6](http://dx.doi.org/10.1016/0031-9422(80)83036-6).
- Hutabarat, O.S., Flachowsky, H., Regos, I., Miosic, S., Kaufmann, C., Faramarzi, S., Alam, M.Z., Gosch, C., Peil, A., Richter, K., Hanke, M.-V., Treutter, D., Stich, K., Halbwirth, H., 2016. Transgenic apple plants overexpressing the chalcone 3-hydroxylase gene of *Cosmos sulphureus* show increased levels of 3-hydroxyphloridzin and reduced susceptibility to apple scab and fire blight. *Planta* 243, 1213–1224. <http://dx.doi.org/10.1007/s00425-016-2475-9>.
- Ibdah, M., Berim, A., Martens, S., Valderrama, A.L.H., Palmieri, L., Lewinsohn, E., Gang, D.R., 2014. Identification and cloning of an NADPH-dependent hydroxycinnamoyl-CoA double bond reductase involved in dihydrochalcone formation in *Malus x domestica* Borkh. *Phytochemistry* 107, 24–31. <http://dx.doi.org/10.1016/j.phytochem.2014.07.027>.
- Ito, T., Fujimoto, S., Shimozaki, M., Taguchi, G., 2014. Production of C-glycosides of flavonoids and related compounds by *Escherichia coli* expressing buckwheat C-glycosyltransferase. *Plant Biotechnol.* 31, 519–524. <http://dx.doi.org/10.5511/plantbiotechnology.14.1016a>.
- Janvier, S., Gosciny, S., Le Donne, C., Van Loco, J., 2015. Low calorie sweeteners in

- food and food supplements on the Italian market. *Food Addit. Contam. Part B* 8, 298–308. <http://dx.doi.org/10.1080/19393210.2015.1094829>.
- Jiang, H., Wood, K.V., Morgan, J.A., 2005. Metabolic engineering of the phenylpropanoid pathway in *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* 71, 2962–2969. <http://dx.doi.org/10.1128/AEM.71.6.2962-2969.2005>.
- Jugd , H., Nguy, D., Moller, I., Cooney, J.M., Atkinson, R.G., 2008. Isolation and characterization of a novel glycosyltransferase that converts phloretin to phlorizin, a potent antioxidant in apple. *FEBS J.* 275, 3804–3814. <http://dx.doi.org/10.1111/j.1742-4658.2008.06526.x>.
- Julleson, D., David, F., Pfeleger, B., Nielsen, J., 2015. Impact of synthetic biology and metabolic engineering on industrial production of fine chemicals. *Biotechnol. Adv.* 33, 1395–1402. <http://dx.doi.org/10.1016/j.biotechadv.2015.02.011>.
- Koeduka, T., Watanabe, B., Suzuki, S., Hiratake, J., Mano, J., Yazaki, K., 2011. Characterization of raspberry ketone/zingerone synthase, catalyzing the alpha, beta-hydrogenation of phenylbutenones in raspberry fruits. *Biochem. Biophys. Res. Commun.* 412, 104–108. <http://dx.doi.org/10.1016/j.bbrc.2011.07.052>.
- Kohlwein, S.D., Eder, S., Oh, C.S., Martin, C.E., Gable, K., Bacikova, D., Dunn, T., 2001. Tsc13p is required for fatty acid elongation and localizes to a novel structure at the nuclear-vacuolar interface in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 21, 109–125. <http://dx.doi.org/10.1128/MCB.21.1.109-125.2001>.
- Koopman, F., Beekwilder, J., Crimi, B., van Houwelingen, A., Hall, R.D., Bosch, D., van Maris, A.J., Pronk, J.T., Daran, J.-M., 2012. *De novo* production of the flavonoid naringenin in engineered *Saccharomyces cerevisiae*. *Microb. Cell Fact.* 11, 155. <http://dx.doi.org/10.1186/1475-2859-11-155>.
- Krivoruchko, A., Nielsen, J., 2015. Production of natural products through metabolic engineering of *Saccharomyces cerevisiae*. *Curr. Opin. Biotechnol.* 35, 7–15. <http://dx.doi.org/10.1016/j.copbio.2014.12.004>.
- Lehka, B.J., Eichenberger, M., Bj rn-Yoshimoto, W.E., Garcia Vanegas, K., Buijs, N., Jensen, N. B., Dyekj r, J. D., Jenssen, H., Simon, E., Naesby, M., 2016. Improving heterologous production of phenylpropanoids in *Saccharomyces cerevisiae* by tackling an unwanted side reaction of Tsc13, an endogenous double bond reductase. Submitted.
- Li, M., Kildegaard, K.R., Chen, Y., Rodriguez, A., Borodina, I., Nielsen, J., 2015. *De novo* production of resveratrol from glucose or ethanol by engineered *Saccharomyces cerevisiae*. *Metab. Eng.* 32, 1–11. <http://dx.doi.org/10.1016/j.ymben.2015.08.007>.
- Lim, E.K., Ashford, D.A., Hou, B., Jackson, R.G., Bowles, D.J., 2004. *Arabidopsis* glycosyltransferases as biocatalysts in fermentation for regioselective synthesis of diverse quercetin glucosides. *Biotechnol. Bioeng.* 87, 623–631. <http://dx.doi.org/10.1002/bit.20154>.
- Luque, A., Sebai, S.C., Santiago-Sch bel, B., Le Coz, Y., Jenot, D., Ramaen, O., Sauveplane, V., Pandjaitan, R., 2014. *In vivo* evolution of metabolic pathways by homologous recombination in mitotic cells. *Metab. Eng.* 23, 123–135. <http://dx.doi.org/10.1016/j.ymben.2014.02.010>.
- Mol, J.N.M., Robbins, M.P., Dixon, R.A., Veltkamp, E., 1985. Spontaneous and enzymic rearrangement of naringenin chalcone to flavanone. *Phytochemistry* 24, 2267–2269. [http://dx.doi.org/10.1016/S0031-9422\(00\)83023-X](http://dx.doi.org/10.1016/S0031-9422(00)83023-X).
- Ninomiya, M., Koketsu, M., 2013. Minor flavonoids (chalcones, flavanones, dihydrochalcones, and aurones). In: *Natural Products Phytochemistry, Botany and Metabolism of Alkaloids, Phenolics and Terpenes*. Springer-Verlag Berlin Heidelberg, 1867–1900. <http://dx.doi.org/10.1038/188440b0>.
- Oka, T., Nemoto, T., Jigami, Y., 2007. Functional analysis of *Arabidopsis thaliana* RHM2/MUM4, a multidomain protein involved in UDP-D-glucose to UDP-L-rhamnose conversion. *J. Biol. Chem.* 282, 5389–5403. <http://dx.doi.org/10.1074/jbc.M610196200>.
- Pandey, R.P., Parajuli, P., Koffas, M.A.G., Sohng, J.K., 2016. Microbial production of natural and non-natural flavonoids: pathway engineering, directed evolution and systems/synthetic biology. *Biotechnol. Adv.* 34, 634–662. <http://dx.doi.org/10.1016/j.biotechadv.2016.02.012>.
- Petersen, C., 1835. Analyse des Phloridzins. *Ann. Pharm.* 15, 178. <http://dx.doi.org/10.1002/jlac.18350150210>.
- Rodriguez, A., Kildegaard, K.R., Li, M., Borodina, I., Nielsen, J., 2015. Establishment of a yeast platform strain for production of *p*-coumaric acid through metabolic engineering of aromatic amino acid biosynthesis. *Metab. Eng.* 31, 181–188. <http://dx.doi.org/10.1016/j.ymben.2015.08.003>.
- Rozmer, Z., Perj si, P., 2016. Naturally occurring chalcones and their biological activities. In: *Phytochemistry Reviews Fundamentals and Perspectives of Natural Products Research*. Springer, Netherlands, 87–120. <http://dx.doi.org/10.1007/s11101-014-9387-8>.
- Scheen, A.J., 2015. Pharmacokinetics, pharmacodynamics and clinical use of SGLT2 inhibitors in patients with type 2 diabetes mellitus and chronic kidney disease. *Clin. Pharmacokinet.* 54, 691–708. <http://dx.doi.org/10.1007/s40262-015-0264-4>.
- Shao, Z., Zhao, H., Zhao, H., 2009. DNA assembler, an *in vivo* genetic method for rapid construction of biochemical pathways. *Nucleic Acids Res* 37, e16. <http://dx.doi.org/10.1093/nar/gkn991>.
- Snijman, P.W., Joubert, E., Ferreira, D., Li, X.C., Ding, Y., Green, I.R., Gelderblom, W.C.A., 2009. Antioxidant activity of the dihydrochalcones Aspalathin and Nothofagin and their corresponding flavones in relation to other rooibos (*Aspalathus linearis*) Flavonoids, Epigallocatechin Gallate, and Trolox. *J. Agric. Food Chem.* 57, 6678–6684. <http://dx.doi.org/10.1021/jf901417k>.
- Song, W.Q., Qin, Y.M., Saito, M., Shirai, T., Pujol, F.M., Kastaniotis, A.J., Hiltunen, J.K., Zhu, Y.X., 2009. Characterization of two cotton cDNAs encoding trans-2-enoyl-CoA reductase reveals a putative novel NADPH-binding motif. *J. Exp. Bot.* 60, 1839–1848. <http://dx.doi.org/10.1093/jxb/erp057>.
- Stahlhut, S.G., Siedler, S., Malla, S., Harrison, S.J., Maury, J., Neves, A.R., Forster, J., 2015. Assembly of a novel biosynthetic pathway for production of the plant flavonoid fisetin in *Escherichia coli*. *Metab. Eng.* 31, 84–93. <http://dx.doi.org/10.1016/j.ymben.2015.07.002>.
- Sun, J., Lin, Y., Shen, X., Jain, R., Sun, X., Yuan, Q., Yan, Y., 2016. Aerobic biosynthesis of hydrocinnamic acids in *Escherichia coli* with a strictly oxygen-sensitive enoate reductase. *Metab. Eng.* 35, 75–82. <http://dx.doi.org/10.1016/j.ymben.2016.02.002>.
- Tsao, R., Yang, R., Young, J.C., Zhu, H., 2003. Polyphenolic profiles in eight apple cultivars using high-performance liquid chromatography (HPLC). *J. Agric. Food Chem.* 51, 6347–6353. <http://dx.doi.org/10.1021/jf0346298>.
- Vrhovsek, U., Masuero, D., Gasperotti, M., Franceschi, P., Caputi, L., Viola, R., Mattivi, F., 2012. A versatile targeted metabolomics method for the rapid quantification of multiple classes of phenolics in fruits and beverages. *J. Agric. Food Chem.* 60, 8831–8840. <http://dx.doi.org/10.1021/jf2051569>.
- Wang, J., Guleria, S., Koffas, M.A., Yan, Y., 2016. Microbial production of value-added nutraceuticals. *Curr. Opin. Biotechnol.* 37, 97–104. <http://dx.doi.org/10.1016/j.copbio.2015.11.003>.
- Watts, K.T., Lee, P.C., Schmidt-Dannert, C., 2004. Exploring recombinant flavonoid biosynthesis in metabolically engineered *Escherichia coli*. *ChemBioChem* 5, 500–507. <http://dx.doi.org/10.1002/cbic.200300783>.
- Werner, S.R., Morgan, J.A., 2009. Expression of a *Dianthus* flavonoid glucosyltransferase in *Saccharomyces cerevisiae* for whole-cell biocatalysis. *J. Biotechnol.* 142, 233–241. <http://dx.doi.org/10.1016/j.jbiotec.2009.05.008>.
- Werner, S.R., Chen, H., Jiang, H., Morgan, J.A., 2010. Enzymatic Synthesis of non-natural flavanones and dihydrochalcones in metabolically engineered yeast. *J. Mol. Catal. B. Enzym.* 66, 257–263. <http://dx.doi.org/10.1016/j.molcatb.2010.05.017>.
- Willits, M.G., Giovanni, M., Prata, R.T.N., Kramer, C.M., De Luca, V., Steffens, J.C., Graser, G., 2004. Bio-fermentation of modified flavonoids: an example of *in vivo* diversification of secondary metabolites. *Phytochemistry* 65, 31–41. <http://dx.doi.org/10.1016/j.phytochem.2003.10.005>.
- Xu, P., Bhan, N., Koffas, M.A.G., 2013. Engineering plant metabolism into microbes: from systems biology to synthetic biology. *Curr. Opin. Biotechnol.* 24, 291–299. <http://dx.doi.org/10.1016/j.copbio.2012.08.010>.
- Yahya, M., Davidovich-Rikanati, R., Eyal, Y., Shechter, A., Marzouk, S., Lewinsohn, E., Ibdah, M., 2016. Identification and characterization of UDP-glucose: Phloretin 4'-O-glycosyltransferase from *Malus x domestica* Borkh. *Phytochemistry* 130, 47–55. <http://dx.doi.org/10.1016/j.phytochem.2016.06.004>.