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Optimizing oleaginous yeast cell factories for flavonoids and hydroxylated flavonoids biosynthesis

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15 ## Abstract

16 Plants possess myriads of secondary metabolites with a broad spectrum of
17 health-promoting benefits. Up to date, plant extraction is still the primary route to produce
18 high-value natural products, which inherently suffers from economics and scalability issues.
19 Heterologous production in microbial host is considered as a feasible approach to overcoming
20 these limitations. Flavonoid and its hydroxylated derivatives represent a diversified family of
21 bioactive compounds, most prominently known as antioxidant and anti-aging agents. Oleaginous
22 yeast is rich in hydrophobic lipid bodies and spatially-organized organelles, which provides the
23 ideal environment for the regioselectivity and stereoselectivity of many plant-specific enzymes.
24 In this report, we validated that *Y. lipolytica* is a superior platform for heterologous production
25 of high-value flavonoids and hydroxylated flavonoids. By modular construction and
26 characterization, we determined the rate-limiting steps for efficient flavonoids biosynthesis in *Y.*
27 *lipolytica*. We evaluated various precursor pathways and unleashed the metabolic potential of *Y.*
28 *lipolytica* to produce flavonoids, including the supply of acetyl-CoA, malonyl-CoA and
29 chorismate. Coupled with the optimized chalcone synthase module and the hydroxylation
30 module, our engineered strain produced 252.4 mg/L naringenin, 134.2 mg/L eriodictyol and
31 110.5 mg/L taxifolin from glucose. Collectively, these findings demonstrate our ability to
32 harness oleaginous yeast as microbial workhorse to expand nature's biosynthetic potential,
33 enabling us to bridge the gap between drug discovery and natural product manufacturing.

34 **Keywords**: Natural products, Flavonoids, Hydroxylation, Metabolic engineering,
35 Oleaginous yeast

36

37 **Running title**: Engineer oleaginous yeast factory for flavonoids

38 ## Introduction

39 Plant possess myriads of secondary metabolites with a broad spectrum of health-promoting
40 benefits. Plant-derived natural products (PNPs) have been used to suppress tumor growth,
41 inhibit retrovirus replication, treat metabolic disease and modulate cholesterol level in both
42 animal and human tests [1]. Up to date, plant extraction is still the primary route to produce PNPs
43 [1]. However, isolation of PNPs from their native sources is limited by low abundance and
44 environmental, seasonal, and regional variations. Total chemical synthesis of complex PNPs
45 often involves toxic catalyst and is commercially unsustainable due to low yield, safety concerns
46 and strict GMP regulations [2]. Genome-mining of plant metabolic pathways [3-8] and reconstruction
47 of biosynthetic gene clusters (BGCs) in industrially-relevant microbes offer significant promise
48 for discovery and scalable synthesis of plant natural products.

49 *E. coli* and *S. cerevisiae* have long been established as host strains to manufacture a large

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50 variety of plant natural products [3, 7-14]. The recent development of oleaginous yeast platforms
51 offers significant advantages over *E. coli* and *S. cerevisiae* (Table 1). The high precursor
52 acetyl-CoA and malonyl-CoA flux along with the hydrophobic lipid bodies make oleaginous
53 yeast a promising host to produce various natural products with complex structures. For example,
54 *Y. lipolytica* is known to internalize substantial portion of carbon feedstock as lipids and fatty
55 acids [15, 16], which provides the ideal amphiphilic environment for the catalytic function of many
56 plant-derived enzymes. It has been recognized as a 'generally regarded as safe' (GRAS)
57 organism for the production of organic acids, polyunsaturated fatty acids (PUFAs) [17, 18] and
58 carotenoids [19-22] in the food and nutraceutical industry. Compared to *S. cerevisiae*, *Y. lipolytica*
59 lacks Crabtree effects, which doesn't require the co-feeding of ethanol for cell to grow. The low
60 pH tolerance [23], strictly aerobic nature [24, 25] and versatile substrate-degradation profile [25-27]
61 enable its robust growth from a wide range of renewable feedstocks. Genetic toolbox
62 development has been expanding to protein expression [28-30], promoter characterization [31-33],
63 YaliBrick-based cloning [34, 35], Golden gate cloning [21, 36], Piggyback transposon [37],
64 genome-editing [34, 38, 39] and iterative gene integration [40], affording us a collection of facile
65 genetic tools for streamlined and accelerated pathway engineering in oleaginous yeast species.

66 Flavonoids represent a diversified family of phenylpropanoid-derived plant secondary
67 metabolites, with an estimated 10,000 unique structures [41, 42]. They are widely found in fruits,
68 vegetables and medicinal herbs and plants. Pharmaceutical studies and animal tests have
69 demonstrated their anti-obesity, anti-cancer, anti-inflammatory, and anti-diabetic activities [43, 44].
70 Flavonoids are among the phytochemicals with proven activity towards the prevention of
71 aging-related diseases, including the treatment of nervous and cardiovascular diseases,
72 Parkinson's and Alzheimer's disease etc. [45]. These health-promoting benefits make flavonoids a
73 distinct family of molecules to fight aging in personal care, nutraceutical industry and clinical
74 trials. Considering the worldwide population with age older than 65 will triple in next 30 years
75 (data from WHO, world health organization), there will be increasing interests and sustainable
76 market demand globally in the near future.

77 To prepare the technological ground and enable microbial synthesis, various flavonoid
78 pathways have been reconstituted in various microbial species including *E. coli* and *S. cerevisiae*
79 recently, and the recombinant production of an array of molecules such as naringenin [46, 47],
80 eriodyctiol [48, 49], resveratrol [50-54], pinocembrin [49, 55], anthocyanins [56-58], quercetin, kaempferol [59],
81 silybin, isosilybin [60], baicalein and scutellarein [61] has been described. Most of the reported
82 studies were centered around simple flavanones or chalcones through enzymatic cascade
83 reactions with the feeding of expensive phenyl precursors (phenylalanine, tyrosine, *p*-coumaric
84 acid and caffeic acid etc), which limits our opportunity for scalable and low-cost production.
85 Structural activity relationship (SAR) studies demonstrate the side chain modifications are
86 highly correlated with flavonoid biological activities [42, 62]. The hydroxylation of flavonoids
87 improves their metabolic stability and membrane permeability, and enhances the solubility and
88 antioxidant property [63]. To date, there remains a significant challenge to produce highly

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89 hydroxylated flavonoids, due to our limited ability to functionally express plant P450
90 hydroxylases and the cytochrome P450 reductases [64]. Oleaginous yeast is rich in membrane
91 structure and subcellular compartments (i.e. lipid bodies, peroxisome, ER and oleosome), which
92 provides the hydrophobic environment that is critical for regioselectivity and stereoselectivity in
93 hydroxylation, glycosylation and prenylation of flavonoids [65-68]. Developing a P450 expression
94 platform in oleaginous yeast will enable us to access the vast majority of complex natural
95 products and deliver robust microbial cell factories to meet the market demand.

96 To bridge this gap, we tested and assessed various plant-derived polyketide synthases, P450
97 monooxygenase/hydroxylases and cytochrome P450 reductases in *Y. lipolytica*, to diversify the
98 structure of flavonoids. With naringenin, eriodyctiol and taxifolin as testing molecules, we
99 characterized the catalytic efficiency of various plant enzymes, including tyrosine ammonia
100 lyase (TAL), 4-coumaroyl-CoA ligase (4CL), chalcone synthase (CHS), chalcone isomerase
101 (CHI), flavonone-3'-hydroxylase (F3'H), flavonol-3-hydroxyalse (F3H) and cytochrome P450
102 reductases. These plant-derived genes were coexpressed with the endogenous acetyl-CoA
103 carboxylase (ACC1) and the pentafunctional AROM polypeptide (ARO1). Systematic pathway
104 debottlenecking indicates that chalcone synthase, ACC1 and cytochrome P450 reductases are the
105 rate-limiting steps for hydroxylated flavonoid production. Specifically, increasing PhCHS copy
106 number and controlling culture pH elevated naringenin production up to 252.4 mg/L. Screening
107 four cytochrome P450 reductases led us to identify that CrCPR derived from *Catharanthus*
108 *roseus* is the most efficient electron shuttle to complete the hydroxylation reaction, despite that
109 endogenous ylCPR1 (YALI0D04422g) displays similar function with relatively low efficiency.
110 Further expression of the plant-derived P450 enzymes, including the flavanol-3' hydroxylase
111 (GhF3'H) from *Gerbera hybrid* led the engineered strain to produce about 110.5 mg/L of
112 taxifolin and 134.2 mg/L of eriodictyol. This work set the foundation for us to engineer
113 oleaginous yeast as chassis for cost-efficient production of flavonoids and hydroxylated
114 flavonoids. The functional expression of plant-derived polyketide synthase, P450
115 monooxygenase and reductases will expand our capability to access nature's biosynthetic
116 potential for drug discovery and natural product manufacturing.

117 # Results and discussion

118 **Modular construction and characterization of flavonoid pathway in *Y. lipolytica***

119 The availability of intracellular malonyl-CoA was reported to be a rate-limiting step of
120 flavonoid synthesis in many microorganisms [69-71]. Considering the high acetyl-CoA and
121 malonyl-CoA flux, we firstly reconstructed the synthetic pathway and validated the feasibility of
122 using *Y. lipolytica* as the chassis to produce flavonoids. In addition, the cytochrome *c* P450
123 (CYP) flavonoid 3'-hydroxylase (F3'H) plays a critical role in oxidizing the phenyl ring and
124 generating hydroxylated flavonoids [72]. Based on the distribution of potential rate-limiting steps,
125 we rationalized and partitioned the flavonoids pathway into two modules, the naringenin

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126 synthesis module (Module I) and the hydroxylation module (Module II) (Fig. 1). Module I
127 contains essential precursor pathway to provide shikimic acid, malonyl-CoA and chalcone
128 precursors; while Module II contains the cytochrome *c* P450 (CYP) flavonoid 3'-hydroxylase
129 (F3'H) and cytochrome *c* P450 reductase (CPR). As a direct assessment of the module efficiency,
130 we have established HPLC method to analyze naringenin, eriodyctiol and taxifolin (Fig. 2).

131 Naringenin is the starting point for many flavonoid functionalization chemistry. We first
132 constructed Module I in *Y. lipolytica* Po1f to synthesize chalcone and naringenin. Because genes
133 from different plants have different specificity and activity, we selected two genes for each of
134 the first three steps in Module I based on the sequence alignment of closely-related plant species.
135 Pathways containing 4CL (*p*-coumaric acid-CoA ligase), CHS (chalcone synthase) and CHI
136 (chalcone isomerase) were assembled in monocistronic forms by YaliBricks cloning platform [34].
137 We observed that all eight constructs containing 4CL, CHS and CHI resulted in the synthesis of
138 naringenin from *p*-coumaric acid, with production ranging from 10 mg/L to 21.5 mg/L (Fig. 3a).
139 Interestingly, the three top producers (Fig. 3a) share the same source of chalcone synthase from
140 *Petunia x hybrid*, indicating that chalcone synthase dictates the efficiency of Module I. To
141 achieve *de novo* synthesis of naringenin, we further introduced tyrosine ammonia-lyase (RtTAL)
142 from *Rhodotorula toruloides*, which has been reported to generate phenylpropanoid precursors
143 from glucose [47, 73]. With the overexpression of RtTAL, we detected *p*-coumaric acid as the direct
144 de-amination product of tyrosine (Supplementary Fig. S1). By complementing the
145 4CL-CHS-CHI pathway, the resulted strain *Y. lipolytica* Po1f/T4SI produced 14.9 mg/L
146 naringenin from glucose (Supplementary Fig. S1). These results validated the feasibility of using
147 *Y. lipolytica* as chassis for *de novo* synthesis of naringenin.

148 There has been a number of reports that *Y. lipolytica* could selectively hydroxylate limonene
149 to perillyl alcohol, perillaldehyde and perillic acids [74, 75], demonstrating the endogenous P450
150 monooxygenase and cytochrome P450 reductase is active enough to hydroxylate methyl group
151 on monoterpenes. Our lab has demonstrated the functional expression of the P450
152 monooxygenase that selectively hydroxylates protodeoxyviolaceinic acid to protoviolaceinic
153 acid, generating the greenish pigment in *Y. lipolytica* [76]. On the basis of these results, we argue
154 that *Y. lipolytic* could be an excellent platform for expression of plant P450 enzymes.

155 F3'H is the critical enzyme involved in the functional hydroxylation of flavonoids.
156 Cytochrome *c* P450 reductase (CPR) is required for electron transfer from NADPH to CYP [77].
157 We have chosen two plant-derived F3'Hs and three CPRs to evaluate which F3'H-CPR pairs
158 could perform hydroxylation chemistry (Fig. 3b). All 6 combinations of F3'H-CPR pairs
159 produced eriodictyol. We observed that strain Po1f/HR with overexpression of CrCPR (derived
160 from *Catharanthus roseus*)[78] coupled with GhF3'H (derived from *Gerbera hybrid*) or GmF3'H
161 (derived from *Glycine max*), led to the highest eriodictyol production around 39 mg/L, with
162 molar conversion yield up to 73.7% from naringenin (Fig. 3b). Interestingly, the two
163 yeast-sourced CPRs, YlCPR from *Y. lipolytica* and ScCPR from *S. cerevisiae* S288c, also gave

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164 rise to eriodictyol, indicating the endogenous CPR is sufficient to shuttle electrons from
165 NADPH to the active oxygen species, which is consistent with the findings reported by Leonard
166 [79]. We further constructed strain *Y. lipolytica* Po1f/HRH with the overexpression of flavanone
167 3-hydroxylase (SlF3H from *Solanum lycopersicum*) and detected about 26.0 mg/L taxifolin with
168 a molar yield of 46.5% from naringenin (Fig. 2). In addition, *Y. lipolytica* endogenous CPR
169 matched F3'H well, with comparable efficiency as CrCPR (Fig. 3b), which is consistent with
170 previous report that *Y. lipolytica* was capable of performing P450-based biotransformation [80, 81].
171 To achieve *de novo* synthesis of eriodictyol and taxifolin, we further complemented the RtTAL
172 with GhF3'H and SlF3H, resulting in strains Po1f/T4SIHR and Po1f/T4SIHRH, respectively.
173 When these strains were tested in shake flask cultures, we obtained 17.2 mg/L eriodictyol and
174 11.3 mg/L taxifolin from glucose, respectively. These results validated that *Y. lipolytica* will be
175 an ideal chassis to functionally express plant P450 enzymes and produce hydroxylated
176 flavonoids.

177 **Tuning gene-copy number to remove pathway bottlenecks**

178 The balance of metabolic flux and mitigation of metabolic burden is a vital factor for
179 optimizing metabolite production in microorganisms [9, 53, 82]. Introduction of large gene cluster
180 may result in the host strain losing cellular fitness when the expression of heterologous proteins
181 exceeds the carrying capacity of the system. For example, metabolic flux improvement by
182 overexpression of upstream pathways may not be accommodated by downstream pathways [82];
183 intermediate accumulation or depletion may reduce cell viability [83]; and overexpressed gene
184 clusters may overload the cell and elicit cellular stress response [84, 85]. We next attempted to probe
185 the rate-limiting steps in Module I and Module II by gradually increasing gene copy number of
186 the genes involved. Gene copy number of each enzymatic step was individually tuned by using
187 YaliBrick assembly platform [34]. Naringenin production increased by 2.64-fold when the gene
188 copy number for chalcone synthase (PhCHS) increased from one to five (Fig. 4a), indicating that
189 CHS is the rate-limiting step in Module I. Increasing the gene copy number of other metabolic
190 genes (RtTAL and Pc4CL) did not have obvious effect on naringenin titer, while increasing the
191 gene copy number of MsCHI decreased naringenin titer by 34.4% (Fig. 4a). We determined that
192 the optimal gene copy number for *PhCHS* is 5, as naringenin production was only marginally
193 increased when the gene copy number was changed from 4 to 5. As larger plasmid may cause
194 genetic instability, we did not further increase the copy number of *PhCHS*.

195 In Module II, increasing one copy number of *CrCPR* resulted in eriodictyol and taxifolin
196 titers increasing by 26.8% and 22.3%, reaching 48.1 mg/L and 31.8 mg/L (Fig. 4b), respectively.
197 Increasing the copy number of *SlF3H* and *GhF3'H* did not have obvious effect on eriodictyol or
198 taxifolin production (Fig. 4b), indicating that CPR is the rate-limiting step in Module II.
199 Eriodictyol and taxifolin titers remained stable, when the gene copy number for *CrCPR* was
200 increased from 2 to 5, suggesting that the optimal ratio of F3'H to its reductase *CrCPR* is 1:2,
201 which is consistent with previous report [86]. The naringenin-to-eriodictyol and taxifolin

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202 conversion ratio reached 90.5% and 56.8%, respectively (Fig. 4b), under the optimal F3'H-CPR
203 ratios. To achieve *de novo* synthesis of eriodictyol and taxifolin, we complemented the
204 eriodictyol and taxifolin pathways with the RtTAL pathway. The resulting strains
205 Po1f/T4S$_{x5}$IHR$_{x2}$ and Po1f/T4S$_{x5}$IHR$_{x2}$H produced 28.9 mg/L eriodictyol and 25.2 mg/L
206 taxifolin from glucose, respectively. These titers are 68.0% and 123.0% higher than the control
207 strains Po1f/T4SIHR and Po1f/T4SIHRH. These results confirmed that tuning gene copy
208 numbers will be a critical step to remove pathway bottlenecks and achieve metabolic balance in
209 genetically modified cell factories, in particularly, oleaginous yeast for flavonoids production.

210 **Improving flavonoid production by enhancing precursor synthesis**

211 We next sought to investigate the upstream shikimic acid and malonyl-CoA pathways to
212 further improve flavonoids production. By supplementing 100 mg/L L-tyrosine with the strain
213 Po1f/T4SI, we observed that naringenin production was increased by 33.6% with glucose as sole
214 carbon source, indicating that upstream shikimic acid pathway is a bottleneck for naringenin
215 synthesis in *Y. lipolytica*. We then overexpressed the pentafunctional polypeptides arom protein
216 ARO1, which catalyzes steps 2 through 6 in the biosynthesis of chorismate, to boost the
217 precursor for L-tyrosine synthesis [87]. YALI0F12639g (YlARO1) is a *Y. lipolytica* homologue of
218 *S. cerevisiae* ARO1 [88], and the DNA sequence for *YlARO1* is composed of 1 intron and 2 exons,
219 encoding a 1556-aa protein. To mitigate unintended mRNA splicing and transcriptional
220 regulation, we removed the internal intron for YlARO1. When this *YlARO1* gene was
221 overexpressed in strain Po1f/T4S$_{x5}$I with optimal Module I settings, naringenin production was
222 increased to 81.6 mg/L, a 50.9% increased compared to the parental strain (Fig. 5a). When we
223 combined Module I with Module II, the resulting strains Po1f/AT4S$_{x5}$IHR$_{x2}$ and
224 Po1f/AT4S$_{x5}$IHR$_{x2}$H produced 40.1 mg/L eriodictyol and 33.4 mg/L taxifolin, which is 38.8%
225 and 32.5% higher than that of the control strains Po1f/T4S$_{x5}$IHR$_{x2}$ and Po1f/T4S$_{x5}$IHR$_{x2}$H,
226 respectively (Fig. 5b).

227 Acetyl-CoA and malonyl-CoA are shared precursors for both lipids and flavonoid pathway
228 [89, 90]. However, malonyl-CoA is primarily used to synthesize lipids cell membrane and support
229 cell growth, leaving only a small amount of acetyl-CoA and malonyl-CoA for heterologous
230 production. To mitigate this competition, it is desirable to redirect the acetyl/malonyl-CoA flux
231 from lipid pathway to flavonoid pathway. Acetyl-CoA carboxylase (ACC) converts acetyl-CoA
232 to malonyl-CoA, which is the first committed step in both lipid and flavonoids biosynthesis [69].
233 In order to enhance intracellular malonyl-CoA synthesis, we screened and tested different ACCs
234 from three sources, including gram-positive bacteria *Corynebacterium glutamicum* ATCC 13032
235 (*CgACC*), gram-negative bacteria *Escherichia coli* MG1655 (Ec_*accABCD*), and *Y. lipolytica*
236 (*YlACC1*, GRYC ID: YALI0C11407g) [91, 92]. Biotin-apoprotein ligase modifies ACC by
237 covalently attaching biotin, which is essential for ACC activity [93]. *EcBirA* and YlBPL1
238 (YALI0E30591g) are *E. coli* and *Y. lipolytica* homologues of biotin-apoprotein ligase,
239 respectively [94, 95]. Genes encoding CgACC, Ec_accABCD, and YlACC1, together with their

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240 biotin-apoprotein ligases were introduced to the naringenin-producing strain. All three ACCs
241 could lead to substantial improvement in naringenin production (Fig. 5a), with *Yl*ACC1
242 demonstrating most obvious effect. For example, overexpression of *YlACC1* in Po1f/AT4S_{x5}I
243 improved naringenin titer by 61.4%, reaching 131.7 mg/L (Fig. 5a). The coupling of
244 *Ec_accABCD* with *EcBirA* also resulted in naringenin production increasing by 22% compared
245 with the strain without *EcBirA* overexpression, indicating the essential role of biotinylation in
246 bacterial ACC activity. This is the first report that *Ec*ACC could be functionally expressed in
247 oleaginous species. Unlike the bacterial ACC, co-expression of *YlACC1* and *YlBPL1* resulted in
248 decreased naringenin production (Fig. 5a). This might indicate the endogenous biotin-apoprotein
249 ligase (YlBPL1) is sufficient to biotinylate ylACC1 in *Y. lipolytica*.

250 We observed that pH value dropped dramatically during the fermentation process (i.e. pH
251 below 3.5 at the end of flask cultivation), and this could be largely ascribed to the overflown
252 metabolism of TCA cycle and respiration [20]. It was recently discovered that acetate secretion
253 was associated with the CoA-transfer reaction between acetyl-CoA and succinate in *Y. lipolytica*,
254 encoded by a mitochondrial enzyme ylACH1 (YALI0E30965) [23]. To recycle acetate, we next
255 sought to overexpress acetyl-CoA synthetases and convert acetate to acetyl-CoA. We tested three
256 acetyl-CoA synthetases from *E. coli*, *S. cerevisiae* and *Y. lipolytica* (Fig. 5a). The native version
257 *YlACS2* demonstrate better effect to recycle acetate. To test the combinatory effects of enhancing
258 chorismate and acetyl/malonyl-CoA precursors, we overexpressed YlARO1 along with
259 YlACS2-YlACC1 in strain Po1f/T4S_{x5}I. The resulting strain produced 149.5 mg/L naringenin,
260 which was 176.3% higher than the titer of the parental strain (Po1f/T4S_{x5}I). We further applied
261 the same strategy to Module II and tested whether overexpression of ARO1, ACC1 and ACS
262 would benefit the accumulation of hydroxylated flavonoids. Overexpression of *YlARO1*
263 increased eriodictyol and taxifolin production by 38.8% and 32.5%, yielding 40.1 mg/L and 33.4
264 mg/L (Fig. 5b), respectively. Overexpressing *YlACS2* and *YlACC1* further increased eriodictyol
265 and taxifolin titers by 41.9% and 52.1%, reaching 56.9 mg/L and 50.8 mg/L, respectively (Fig.
266 5b). Due to the large size of the plasmid construct (more than 40 kb), we did not further pursue
267 the synergistic effect of ARO1, ACC1 and ACS2 in the current work. These results indicated that
268 manipulation of acetyl-CoA, malonyl-CoA and chorismate pathway was critical to improve
269 flavonoid production in *Y. lipolytica*.

270 **Boosting flavonoid production by bioprocess optimization**

271 The C/N ratio is an important factor for regulating the acetyl-CoA and NADPH fluxes in
272 *Yarrowia lipolytica* [33]. It has been reported that nitrogen starvation triggers the repression of
273 TCA cycle and induces lipogenesis in oleaginous species [96, 97]. It was recently discovered that
274 C/N ratio dynamically regulates lipogenic promoter activity in *Y. lipolytica* [33]. In this study, C/N
275 ratio was optimized in two patterns to improve flavonoid synthesis, by either adjusting the
276 amount of nitrogen source (ammonia sulfate) or carbon source (glucose). The results showed
277 that altering $(NH_4)_2SO_4$ content did not have obvious effect on naringenin titer. Slightly higher

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278 naringenin titer was achieved at higher C/N ratio (C/N=120) (Supplementary Fig. S2a). On the
279 contrary, it was clearly shown that higher C/N ratio was advantageous to improving naringenin
280 titer by increasing the level of glucose. Specifically, naringenin titer was increased about 56%
281 when the C/N was altered from 40 to 160 (Supplementary Fig. S2b). Since glucose is the direct
282 precursor for chorismate and malonyl-CoA, it indicates that there is still much space to further
283 improve the precursor flux in *Y. lipolytica*.

284 In order to produce flavonoids with inexpensive YPD (yeast extract, peptone and dextrose)
285 medium, we integrated the optimized pathways into *Y. lipolytica* Po1f genome with our recently
286 developed integration methods [98]. The best-performing strains NarPro/ASC, ErioPro, and
287 TaxiPro produced 71.2 mg/L naringenin, 54.2 mg/L eriodictyol, and 48.1 mg/L taxifolin in YPD
288 medium, respectively [40]. We observed that the pH dropped to 3.2 at the end of the fermentation
289 in YPD, possibly due to the accumulation of various organic acids [20]. We next sought to buffer
290 the media pH by using either phosphate buffer saline (PBS) or calcium carbonate ($CaCO_3$).
291 Supplementation of 4% $CaCO_3$ maintained stable pH and improved naringenin titer by 31.2%,
292 reaching 138.1 mg/L at 144 h, while PBS buffer did not have obvious effect compared with the
293 control (Supplementary Fig. S4). We also analyzed the combinatory effects of inhibiting fatty
294 acid synthesis by adding cerulenin [99], maintaining stable pH, and supplying sodium acetate. The
295 fermentation time course showed that we could achieve steady improvement in naringenin
296 production (Supplementary Fig. S5). For example, supplementation of 1 mg/L cerulenin with 40
297 g/L $CaCO_3$ further improved naringenin titer by 31.2%, (Supplementary Fig. S5c). However,
298 supplying 5 mM NaAc did not result in further increase in naringenin production
299 (Supplementary Fig. S5d). By intermittently feeding glucose after 48 hours, the
300 chromosomally-integrated strain produced 252.4 mg/L naringenin under optimal conditions (Fig.
301 6a). Likewise, we tested the eriodictyol and taxifolin production in YPD medium by buffering
302 media pH with 40 g/L $CaCO_3$ and inhibiting fatty acid synthesis with 1 mg/L cerulenin. We
303 observed that strains ErioPro and TaxiPro produced 95.5 mg/L eriodictyol and 79.1 mg/L
304 taxifolin in 144 h, which were 76.2% and 64.4% higher than that without $CaCO_3$ and cerulenin.
305 ErioPro and TaxiPro produced 134.2 mg/L eriodictyol and 110.5 mg/L taxifolin at the end of the
306 fermentation process (Fig. 6b). These results indicate that *Y. lipolytica* is an ideal platform to
307 functionally express plant-derived P450 enzymes. By optimizing the bioprocess, we could
308 substantially improve the titer of naringenin, eriodictyol and taxifolin in
309 metabolically-engineered oleaginous yeast species.

310 Conclusions

311 The heterologous production of hydroxylated flavonoids remains a challenging task; and
312 only limited successful pathway engineering endeavors have been reported to date. Oleaginous
313 yeast is rich in lipid and internal membrane structures, which provides the hydrophobic lipid
314 environment and spatially-organized organelles that are critical for plant P450 enzyme

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315 functionality. In this report, we validated that *Y. lipolytica* is a superior platform for heterologous
316 production of high value flavonoids and hydroxylated flavonoids. By modular construction and
317 characterization of various genes involved in plant flavonoid biosynthesis, we determined that
318 chalcone synthase (CHS), flavanone 3-hydroxylase (F3H) and cytochrome c P450 reductase
319 (CrCPR) were the critical steps to engineer flavonoid production in *Y. lipolytica*. Coupling with
320 the upstream amino acid degradation pathway (tyrosine ammonia lyase from *Rhodotorula*
321 *glutinis*), for the first time, we achieved *de novo* production of naringenin, eriodictyol and
322 taxifolin from glucose in *Y. lipolytica*. By using a modular cloning platform to assemble multiple
323 genetic constructs, we further determined the optimal gene copy ratio for CHS, F3H and CrCPR
324 to cooperatively improve flavonoids and hydroxylated flavonoids production. We then unleashed
325 the metabolic potential of the yeast host by screening and testing a number of precursor
326 pathways, including the acetyl-CoA synthetase, acetyl-CoA carboxylase and chorismate pathway
327 (the pentafunctional AROM polypeptide ARO1). Coupled with the optimized chalcone synthase
328 module and the hydroxylation module, our engineering strategies synergistically removed
329 pathway bottlenecks and led to a 15.8-fold, 6.9-fold and 8.8-fold improvement in naringenin,
330 eriodictyol and taxifolin production, respectively. Collectively, these findings demonstrate our
331 abilities to harness oleaginous yeast as microbial workhorse to expand nature's biosynthetic
332 potential, which allows us to produce complex natural products from cheap feedstocks.

333 Materials and methods

334 Genes, plasmids, and strains

335 Genes encoding *Rhodotorula R. toruloides* tyrosine ammonia lyase (*RtTAL*), *Petroselinum*
336 *crispum* (parsley) 4-coumarate-CoA ligase (*Pc4CL*), *Petunia x hybrid* chalcone synthase
337 (*PhCHS*), *Medicago sativa* chalcone isomerase (*MsCHI*), *Escherichia coli* acetyl-CoA
338 synthetase (*EcACS*), and *Corynebacterium glutamicum* ATCC 13032 acetyl-CoA carboxylase
339 (*CgACC*) were frozen stocks of our laboratory. Genes encoding *Solanum lycopersicum*
340 4-coumarate-CoA ligase (*Sl4CL*), *Hordeum vulgare* chalcone synthase (*HvCHS2*), *Petunia x*
341 *hybrid* chalcone isomerase (*PhCHI*), *Gerbera hybrid* flavonoid 3'-hydroxylase (*GhF3'H*),
342 *Glycine max* flavonoid 3'-hydroxylase (*GmF3'H*), *Catharanthus roseus* cytochrome P450
343 reductase (*CrCPR*), and *Solanum lycopersicum* flavanone 3-hydroxylase (*SlF3H*) were
344 optimized and synthesized by GenScript (Nanjing, China). Genes encoding *Yarrowia lipolytica*
345 pentafunctional arom protein (*YlARO1*), *Yarrowia lipolytica* cytochrome P450 reductase
346 (*YlCPR*), *Yarrowia lipolytica* acetyl-CoA synthetase (*YlACS2*) were amplified from *Yarrowia*
347 *lipolytica* Po1f genomic DNA by PCR. *Saccharomyces cerevisiae* cytochrome P450 reductase
348 (*ScCPR1*) and *Saccharomyces cerevisiae* acetyl-CoA synthetase (*ScACS2*) were amplified from
349 *Saccharomyces cerevisiae* genomic DNA by PCR. Genes used in this project were listed in
350 Supplementary Table S1.

351 Plasmid pYLXP' was a stock of our laboratory [89]. Plasmid pYLXP'2 was constructed by

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352 replacing *LEU2* marker with *URA3* marker. Both pYLXP' and pYLXP'2 were YaliBrick
353 plasmids and used for flavonoid pathway construction [34]. *Escherichia coli* (*E. coli*) NEB 5α was
354 used for plasmid construction, propagation, and maintenance. *Yarrowia lipolytica* (*Y. lipolytica*)
355 Po1f (ATCC MYA-2613, MATA ura3-302 leu2-270 xpr2-322 axp2-deltaNU49 XPR2::SUC2)
356 was used as the chassis to construct flavonoid pathways.

357 To achieve *de novo* synthesis of eriodictyol and taxifolin, we transformed
358 pYLXP'2-GhF3'H-CrCPR and pYLXP'2-GhF3'H-CrCPR-SlF3H into Po1f/T4SI, resulting in
359 Po1f/T4SIHR and Po1f/T4SIHRH, respectively. The strain containing 5 copies of *PhCHS* was
360 named as Po1f/T4S$_{x5}$I. We chose to use the plasmids pYLXP'2-HR$_{x2}$ and pYLXP'2-HR$_{x2}$H,
361 which contain 2 copies of *CrCPR*, to construct eriodictyol and taxifolin pathways. To achieve *de*
362 *novo* synthesis of eriodictyol and taxifolin, strains Po1f/T4S$_{x5}$IHR$_{x2}$ and Po1f/T4S$_{x5}$IHR$_{x2}$H
363 were constructed by transforming plasmids pYLXP'2-HR$_{x2}$ and pYLXP'2-HR$_{x2}$H into strain
364 Po1f/T4S$_{x5}$I, respectively. We over-expressed YlARO1 along with YlACS2-YlACC1 in strain
365 Po1f/T4S$_{x5}$I, and name the new strain as Po1f/AT4S$_{x5}$I-YlACS2-YlACC1. By introducing
366 Module II into strains Po1f/AT4S$_{x5}$I and Po1f/AT4S$_{x5}$I-YlACS2-YlACC1, we obtained
367 eriodictyol producing strains Po1f/AT4S$_{x5}$IHR$_{x2}$ and Po1f/AT4S$_{x5}$IHR$_{x2}$-YlACS2-YlACC1 and
368 taxifolin producing strains Po1f/AT4S$_{x5}$IHR$_{x2}$H and Po1f/AT4S$_{x5}$IHR$_{x2}$H-YlACS2-YlACC1.
369 Strains constructed in this project were listed in Supplementary Table S2.

370 **Pathway construction**

371 Genes *RtTAL*, *Pc4CL*, *PhCHS*, *MsCHI*, *EcACS*, *CgACC*, *YlCPR*, *YlACS2*, *ScCPR1*, and
372 *ScACS2* were amplified using respective primers listed in Supplementary Table S3. The PCR
373 product was assembled with *SnaBI* digested pYLXP' or pYLXP'2 using Gibson Assembly
374 method. *YlARO1* is composed of 2 extrons and 1 intron. The extrons were amplified by using
375 primer pairs ARO1_up F/ARO1_up R and ARO1_down F/ARO1_down R respectively. The
376 resulting PCR products were assembled with *SnaBI* digested pYLXP' to yield pYLXP'-ARO1,
377 removing the intron sequence. For gene expression, the start codon was removed and a nucleic
378 acid sequence "TAACCGCAG" was added at the upstream of coding gene to complete the
379 intron [34].

380 The YaliBrick method was used to assemble the synthetic pathways [34]. pYLXP' derived
381 plasmids were used to assemble the pathways of Module I, while pYLXP'2 derived plasmids
382 were used to assemble the pathways of Module II and ACS and ACC. Generally, the donor
383 plasmids were digested with *Avr*II/*Sal*I, and the destination plasmids were digested with
384 *Nhe*I/*Sal*I. The resulting plasmids containing monocistronic configurations were obtained by T4
385 ligation. For the assemble of genes containing any of these isocaudomers, other isocaudomers
386 were used. Specifically, the donor plasmid pYLXP'-YlARO1 was digested with *Hpa*I/*Nhe*I, and
387 the destination plasmid pYLXP'-T4S$_{x5}$I was digested with *Hpa*I/*Avr*II. The resulting plasmid
388 pYLXP'-AT4S$_{x5}$I was obtained by inserting *YlARO1* into pYLXP'-T4S$_{x5}$I using T4 ligation. The
389 donor plasmid pYLXP'2-HR$_{x2}$H was digested with *Cla*I/*Nhe*I, and the destination plasmid

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390 pYLXP'2-ScACS2-YlACC1 was digested with *Cla*I/*Avr*II. The resulting plasmid
391 pYLXP'2-HR$_{x2}$H-ScACS2-YlACC1 was obtained by inserting genes GhF3'H-CrCPR$_{x2}$-SlF3H
392 into pYLXP'2-ScACS2-YlACC1 using T4 ligation. Plasmids pYLXP'2-YlACC1,
393 pYLXP'2-EcACCABCD-EcBirA, and pYLXP'2-YlBPL1 were frozen stocks of our laboratory
394 [34]. Plasmids used in this paper were listed in Supplementary Table S4.

395 **Yeast transformation and screening**

396 The lithium acetate (LiAc) method was used for the transformation. *Y. lipolytica* was
397 cultured on YPD plate at 30°C for 16-22 h. The transformation solution was prepared as follows:
398 90 μL 50% PEG4000, 5 μL 2 M LiAc, 5 μL boiled single strand DNA (salmon sperm,
399 denatured), and 200-500 ng plasmid DNA. The transformation solution was mixed well by
400 vortexing before use. Next, the yeast was transferred to the transformation solution, and mixed
401 well by vortexing for at least 10 seconds. The transformation mixtures were then incubated at
402 30˚C for 30-45 min. The transformation mixture was then vortexed for 15 seconds every 10
403 minutes, followed by an additional 10 min heat shock at 39°C to increase transformation
404 efficiency. For the transformation of pYLXP' and derivative plasmids, the mixture was plated on
405 leucine drop-out complete synthetic media (CSM-Leu). For the transformation of pYLXP'2 and
406 derivative plasmids, the mixture was plated on uracil drop-out complete synthetic media
407 (CSM-Ura). For the transformation of both plasmids, the mixture was plated on leucine and
408 uracil drop-out complete synthetic media (CSM-Leu-Ura). Strains NarPro/ASC, ErioPro, and
409 TaxiPro were constructed in previous work [40]. Strains used in this paper were listed in Strains
410 constructed in this project were listed in Supplementary Table S2.

411 **Cultivation and pH control**

412 The seed was cultured in regular leucine, or uracil, or leucine and uracil drop-out complete
413 synthetic media (CSM-Leu, or CSM-Ura, or CSM-Leu-Ura) at 30°C for 2 days. The seed culture
414 was inoculated to 25 mL nitrogen-limited media (C/N = 80) to a final concentration of 2% (v/v).
415 The fermentation was carried out in 250 mL shak flask at 30°C 220 rpm. C/N ratio was
416 optimized by two patterns: i) fixing glucose content (40 g/L) and altering $(NH_4)_2SO_4$ content; ii)
417 fixing $(NH_4)_2SO_4$ content (0.73348 g/L) and altering glucose content. To analyze the effect of
418 cerulenin, oleic acid, and sodium acetate (NaAc) on flavonoid synthesis, a final concentration of
419 5 g/L oleic acid or 1 mM NaAc was added at the starting point, while a final concentration of 1
420 mg/L cerulenin was added at 48 h. To buffer the acidity, 20 mM phosphate buffer saline (PBS,
421 Na_2HPO_4-NaH_2PO_4) or 40 g/L $CaCO_3$ was used respectively. In the fed-batch fermentation, the
422 starting glucose concentration was 40 g/L, and a final concentration of 10 g/L glucose was added
423 every 24 h from 48 h.

424 **Analytical methods**

425 Samples were taken at 144 h. In the fed-batch fermentation, samples were taken every 24 h.
426 For naringenin, eriodictyol, and taxifolin analysis, samples were diluted in methanol; whole for

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427 glucose analysis, samples were diluted in H_2O. Samples were shaken with glass beads to release
428 the metabolites for analysis. Naringenin, eriodictyol, taxifolin, and glucose were analyzed using
429 Agilent HPLC 1220 as previously described [40].

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438 Author contributions

439 PX and JZ conceived the topic. YL performed genetic engineering and fermentation experiments.
440 YL and PX wrote the manuscript. JZ and MK revised the manuscript.

441 Conflicts of interests

442 A provisional patent has been filed based on the results of this study.

443

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704 # Tables

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***Table 1.* Comparison of *E. coli*, *S. cerevisiae* and *Y. lipolytica* as chassis to produce plant natural products**

Expression platform	*E. coli*	*S. cerevisiae*	*Y. lipolytica*
Genetic tools	+++++	+++++	+++
Genome annotation	+++++	++++	++++
Acetyl-CoA/Malonyl-CoA/HMG-CoA flux	++	+++	+++++
P450 expression	+	+++	+++++
Substrate flexibility	++++	++	++++
Acid tolerance	+++	++	+++++
FDA safety	+	+++++	+++++
Hydrophobic lipid body environment	+	++	+++++

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707 # Figure and legends

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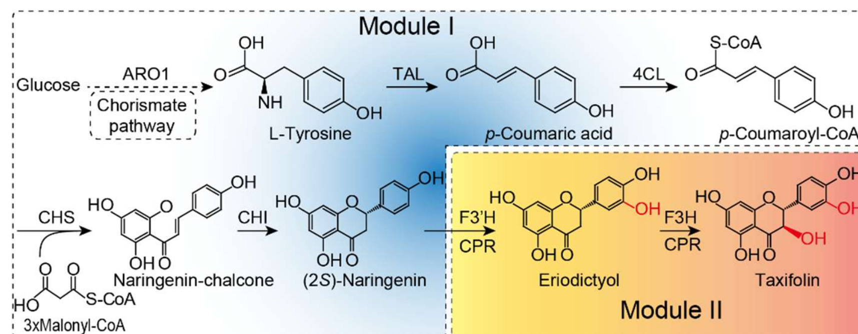

710

711 **Fig. 1.** Modular strategy to optimize naringenin, eriodictyol and taxifolin pathways. Based on
712 the reaction cascades, flavonoid pathway was partitioned into 2 modules, naringenin synthetic
713 module (Module I) and hydroxylation module (Module II). Module I contains chorismate
714 pathway and malonyl-CoA utilizing step, and Module II contains flavanone 3-hydroxyase and
715 cytochrome c P450 reductases.

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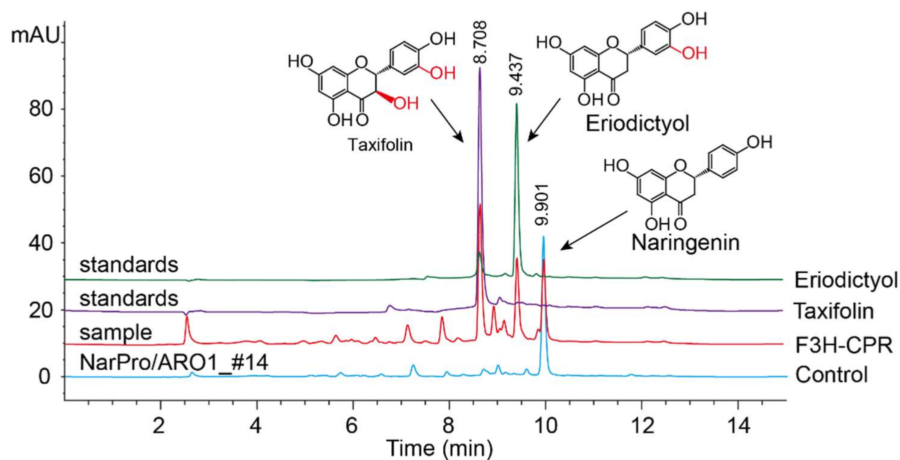

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720 **Fig. 2.** HPLC profile of naringenin, eriodictyol and taxifolin. Two hydroxylated flavonoid
721 standards (taxifolin, purple and eriodictyol, green) were injected to HPLC. One
722 naringenin-producing sample (blue) and one taxifolin-producing sample (red) are shown in the
723 chromatogram.

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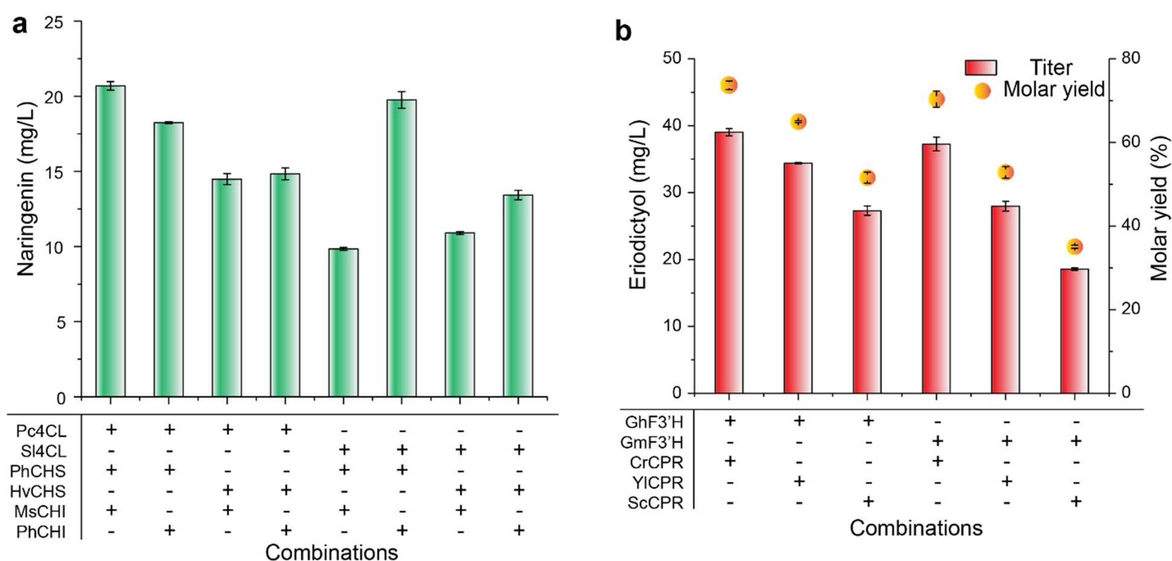

727

728 **Fig. 3.** Screening of gene combinations for improving flavonoid production. **(a)** Screening of
729 *4CH*, *CHS* and *CHI* genes from different pants for naringenin production. **(b)** Screening of *F3'H*
730 and *CPR* genes from different organisms for eriodictyol production. Plant name and gene
731 sources can be found in supplementary table S1.

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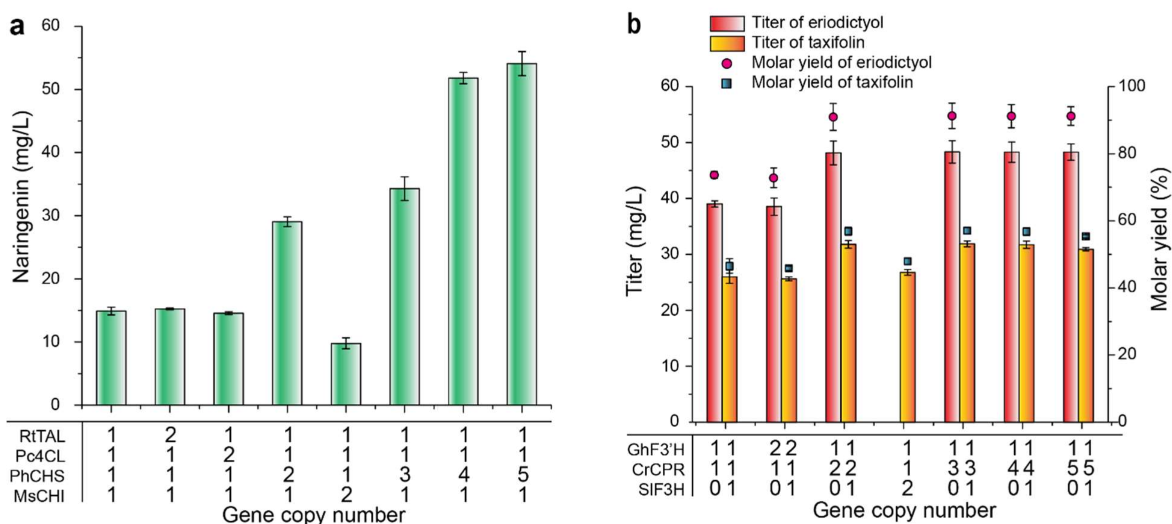

749

750 **Fig. 4.** Overcoming rate-limiting steps by tuning gene copy numbers. Rate-limiting steps were
751 determined by gradually increasing gene copy number of each step. Numbers refer to gene copy
752 numbers. **(a)** Rate-limiting step analysis and optimization of module I to improve naringenin
753 production. **(b)** Rate-limiting step analysis and optimization of module II to improve eriodictyol
754 and taxifolin production. The molar yield was calculated using 50 mg/L naringenin as feeding
755 substrate. The number 0 refers to the module that does not contain the respective gene.

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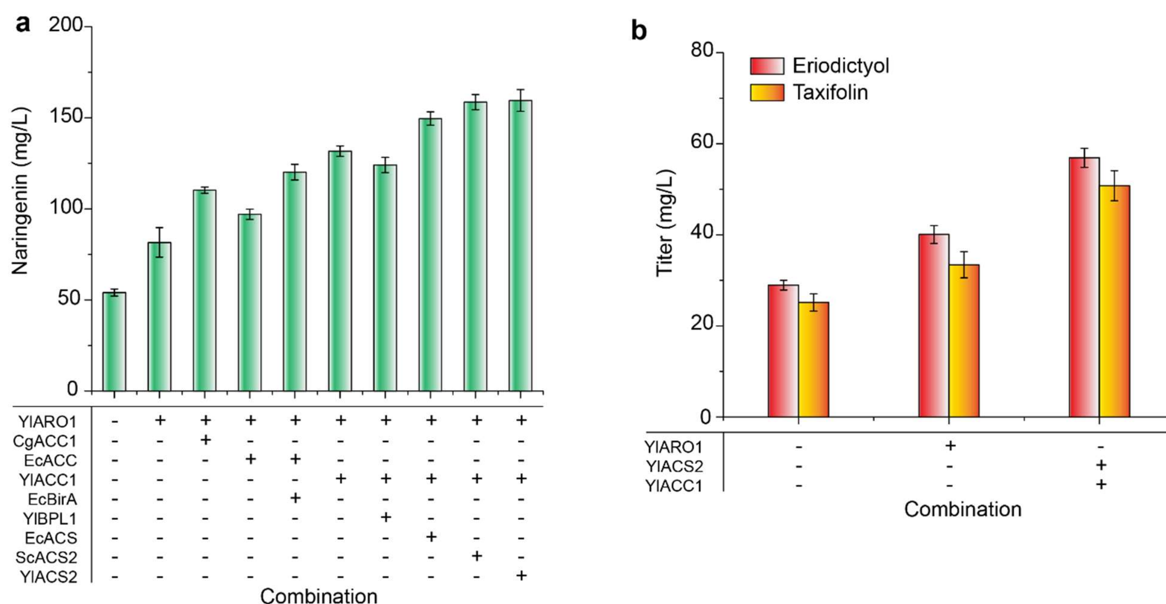

760

761 **Fig. 5.** Improving naringenin, eriodictyol, and taxifolin production by enhancing precursor
762 synthesis. **(a)** Identification of possible rate-limiting steps by overexpression of chorismate
763 pathway (ARO1), malonyl-CoA pathway (ACC) and acetyl-CoA pathway (ACS). The related
764 genes were overexpressed in strains Po1f/T4S$_{x5}$I. **(b)** Effects of improving malonyl-CoA and
765 chorismate synthesis on eriodictyol and taxifolin production. For eriodictyol production, the
766 related genes were overexpressed in Po1f/T4S$_{x5}$IHR$_{x2}$. For taxifolin production, the related
767 genes were overexpressed in Po1f/T4S$_{x5}$IHR$_{x2}$H. + referred to the presence of gene
768 overexpression. – referred to the absence of gene overexpression.

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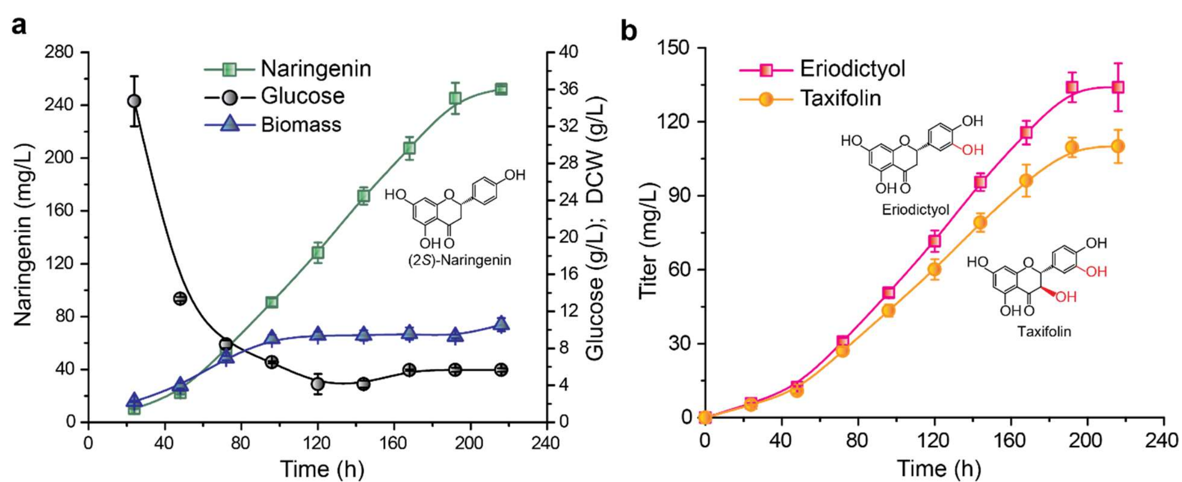

783

784 **Fig. 6.** Naringenin, eriodictyol and taxifolin production under the optimal conditions. (**a**).
785 Naringenin production; (**b**) Eriodictyol and taxifolin production. The engineered strains were
786 cultivated in fed-batch fermentation and buffered with 40 g/L $CaCO_3$. A final concentration of 1
787 mg/L cerulenin was supplemented at 48 h to inhibit fatty acid synthesis.

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