Development of a growth coupled dynamic regulation network balancing malonyl-CoA node to enhance (2S)-naringenin synthesis in *E. coli*

Shenghu Zhou^{1,2,#}, Shuo-Fu Yuan^{3,#}, Priya H Nair⁴, Hal S Alper^{3,4}, Yu Deng^{1,2,*}, Jingwen Zhou^{1,2,*}

¹ National Engineering Laboratory for Cereal Fermentation Technology (NELCF), Jiangnan University, 1800 Lihu Road, Wuxi, Jiangsu 214122, China;

² Jiangsu Provincial Research Center for Bioactive Product Processing Technology, Jiangnan University, 1800 Lihu Road, Wuxi, Jiangsu 214122, China;

³ Institute for Cellular and Molecular Biology, The University of Texas at Austin, 2500 Speedway Avenue, Austin, Texas 78712;

⁴ McKetta Department of Chemical Engineering, The University of Texas at Austin, 200 E Dean Keeton St. Stop C0400, Austin, Texas 78712;

Mailing address: National Engineering Laboratory for Cereal Fermentation Technology, 1800 Lihu Road, Wuxi, Jiangsu 214122, China.

Phone: +86-85914371, Fax: +86-510-85914371

* Correspondence should be addressed to Yu Deng and Jingwen Zhou

E-mail: dengyu@jiangnan.edu.cn, zhoujw1982@jiangnan.edu.cn

Shenghu Zhou and Shuo-Fu Yuan contributed equally.

Abstract:

Generally, high- and low-performance nongenetic variants and young and aged cells co-existed in culture at all growth phases. In this regard, individually and dynamically regulating the metabolic flux of single cells based on their cellular state is highly useful for improving the performance of populations. However, balancing the trade-offs between biomass formation and compound over-production requires sophisticated dynamic regulation networks (DRNs) which can be challenging. Here, we developed a growth coupled NCOMB (Naringenin-Coumaric acid-Malonyl-CoA-Balanced) DRN with systematic optimization of (2*S*)-naringenin and *p*-coumaric acid-responsive regulation pathways for real-time control of intracellular supply of malonyl-CoA. In doing so, the acyl carrier protein was used as a new critical node for fine-tuning malonyl-CoA consumption instead of fatty acid synthase. Following directed evolution of the NCOMB DRN, we obtained a strain with cumulative 8.4-fold improvement in (2*S*)-naringenin production with balanced cell growth, demonstrating the high efficiency of this system for improving pathway production.

Keywords: Dynamic regulation network, (2*S*)-naringenin, *p*-coumaric acid, malonyl-CoA, acyl carrier protein, directed evolution

1 Introduction

Microbial production of value-added biochemicals often requires the development of a robust, efficient, and high-performance strain. To this end, static metabolic engineering strategies including multi-module optimization (1), promoter and RBS engineering (2), and CRISPR/Cas9-based genome engineering (3) are commonly employed for the rewiring of metabolic flux to maximize production. Despite significant successes in the literature and industry using these static strain engineering paradigms, it remains difficult to balance the trade-offs between growth and production. To address this balance within static strain engineering approaches, researchers often utilize culture-level changes including dynamic environmental changes such as incubation temperature, osmolarity, and nutrient availability or oscillating levels of intermediate metabolites (4). As an alternative, genetic approach, the use of transcription factor-based biosensors to monitor cell growth and/or metabolism provides the potential to enable a responsive mode to metabolism termed dynamic metabolic regulation (5).

Using the principles of dynamic regulation, a series of control systems (typically enabled by biosensors) fine-tune the cells to synthesize a target compound at maximum production levels (4). These control systems can be dynamically regulated at either the environmental or metabolic level. Generally, biosensors that could respond to universal, environmental induction factors such as temperature (6), dissolved oxygen (5), and light (7) enable construction of environment-responding dynamic regulation networks (DRNs). For example, Zhao *et al.* established a blue light inducible transcription factor (VP16-EL222) controlled OptoEXP system for isobutanol production and demonstrated a 4-fold improvement in the titer compared with the strain lacking dynamic regulatory circuits (8). Likewise, temperature is a common environment inducer. Using the promoter P_R/P_L system along with the repressor protein CI857, temperature was able to dynamically control the expression of isocitrate dehydrogenase (*icd*) thus achieving a balance between itaconic acid biosynthesis and cell growth (9). The cell factories constructed in these studies respond to

environment changes, however, environmental signals represent the integration of cellular state and thus not explicitly reflect the differences between intracellular states.

More specifically, fermentation conductions such as cell density represent the overall state of the cell population. In reality, a culture contains a mixture of high- and low-performance nongenetic variants and young and aged cells in all growth phases (10). As a result, precisely regulating metabolic flux based on the intracellular state can help improve overall productivity within a fermentation. To this end, it is important to identify biosensors that can directly monitor the intracellular metabolic state of single cells and thus individually regulate gene expression within single cells in response to a critical metabolite level (4). Recent examples of this strategy have been used to balance malonyl-CoA as it serves as a central metabolic precursor. For example, by coupling the glucose-responsive promoter (P_{HXT1}) with a malonyl-CoA sensitive transcriptional repressor (FapR), David *et al.* constructed a DRN to drive the flux from the malonyl-CoA pool toward 3-hydroxypropionic acid formation, leading to a 10-fold increase in titer (11). Additionally, Xu *et al.* developed an oscillator to dynamically balance malonyl-CoA pool through controlling the expression of acetyl-CoA carboxylase (ACC) and fatty acid synthase (12). In their report, the best producer displayed a 2.1-fold improvement in fatty acids titer (3.86 g/L) (13). While successful in improving product titers, metabolite-responsive DRNs only monitor the fluctuating level of a metabolite without coupling with the growth profile of single cells, usually at the expense of growth.

In contrast to these approaches, the work present here demonstrates a dynamic optimization coupling that utilizes a growth coupled single cell sensing and regulating DRNs cascade to improve the production of the flavonoid (2S)-naringenin. Specifically, this DRN cascade focuses on malonyl-CoA, a crucial metabolite used as a carbon-chain elongation unit for important compounds such as flavonoids (14), antibiotics (15), and fatty acids (12). However, the availability of intracellular malonyl-CoA is limited and reduces from 0.23 to 0.01 nmol/(mg dry wt) when cells

transit from exponential to stationary stage (16). Traditional approaches to increase malonyl-CoA content usually represses the fatty acid biosynthetic pathway (17). However, excessive repression of this pathway leads to impaired cell growth. Additionally, oversupply of malonyl-CoA causes an undesired malonylation of the proteome, thus establishing a further carbon burden in engineered *E. coli*. (18). As a result, malonyl-CoA must be in an exquisite balance within cells. In this study, we designed and optimized a metabolite-responsive NCOMB (Naringenin-Coumaric acid-Malonyl-CoA-Balanced) DRN which can real-time monitor the concentration of (2S)-naringenin and *p*-coumaric acid to dynamically enhance the production of malonyl-CoA at all growth phases. The designed DRN includes three modules: (1) module A that functionally produces (2S)-naringenin, (2) module B that dynamically amplifies the metabolic flux toward (2S)-naringenin synthetic pathway with coupling cell growth, and (3) module C that dynamically responds to *p*-coumaric acid and enhances the production of malonyl-CoA during late stages of fermentation (**Fig. 1**). In doing so, we successfully demonstrate the ability of NCOMB DRN to achieve the single cell dynamic regulation and achieved a balance of (2S)-naringenin production as well as cell growth based on the level of intracellular metabolite, which could further facilitate the advancement of DRN design.

2 Results

2.1 Construction and optimization of critical DRN biosensors

The (2S)-naringenin (FdeR based)- (19, 20) and *p*-coumaric acid (PadR based)- (21) responsive biosensors are the central regulatory elements of our NCOMB DRN. To fine-tune the transcription of downstream target genes, it was necessary to engineer a moderate responsiveness and tight control of these elements. Although the responsive promoter architecture of P_{fdeA} (19) and P_{padC} (22, 23) have been reported, information of the corresponding promoter strength is still unclear. Given that the length of 5'-UTR region for the P_{fdeA} and P_{padC} could change the secondary structure

of anti-senseRNA and affect the expression of target genes in our DRN design (**Fig. 1**), we first generated a series of truncated promoters to thoroughly investigate their impacts on the expression of corresponding genes of interest.

P_{fdeA} is a bidirectional promoter 288 bp in length and contains a dozen FdeR binding sites (T-N11-A) spread over a wide region (19). To dissect function of this element, we truncated P_{fdeA} in both directions based on the position of FdeR binding sites and generated 8 variants of P_{fdeA} (M-N) promoter actuated biosensors (**Fig. 2a and Supplementary Fig. 1a**). As a result, we identified that the shorter version of P_{fdeA} (223-135), only 88 bp in length, exhibited around 4-fold higher signal-to-noise ratio than that of the original P_{fdeA} (288-1) when induced by 50 mg/L of (2*S*)-naringenin (**Fig. 2b and c**). Furthermore, the sensor with the truncated promoter P_{fdeA} (223-135) displayed a higher sensitivity over other constructs (**Supplementary Fig. 1b**). These properties can enable the newly designed (2*S*)-naringenin-responsive biosensor to express anti-sense RNAs and proteins (**Fig. 1**) at a very low level of (2*S*)-naringenin.

Turning to the second promoter system, P_{padC} , used to control the expression of *yveF*, *yveG*, and *padC* in an operon form, we predicted that the position of potential -35/-10 boxes by BDGP neural network promoter prediction software v2.2 (24) and identified 7 candidates sites (**Fig. 2d**). In a similar fashion with P_{fdeA} , we established a promoter truncation series for P_{padC} based on the position of predicted -35/-10 boxes. Using the truncated promoter P_{padC} (P-Q), 8 variants of *p*-coumaric acid and IPTG-responsive biosensors were constructed and evaluated (**Fig. 2e and Supplementary Fig. 1c**). As shown in **Fig. 2f**, the position of 608-476 was identified as a suitable region for protein expression, which demonstrated a 4.2-fold higher expression than that of the original P_{padC} (608-1). Furthermore, the shortest version (608-496) without the RBS exhibited an ideal expression strength, 2.3-fold of that of the original P_{padC} (608-1), which could be also used for expression of anti-sense RNA. To avoid the leaky expression and expensive inducer inherent in T7-based inducible control, we next employed a exponential phase-induced promoter P_{cspA} (25) to

control the expression of PadR (**Fig. 2g**). Compared with the T7 promoter, P_{cspA} exhibited lower background with only 7.2% of that when 5 μ M IPTG was added to the medium (**Fig. 2h**). Despite the lower total responsive level of P_{cspA} (only 33.3% the level of 5 μ M IPTG-induced T7 controlled biosensor (**Fig. 2h**)), the dynamic range of *p*-coumaric acid response was 18.2-fold higher in this system compared with the T7 controlled biosensor system. In addition, previous study has demonstrated that the expression level of P_{cspA} in exponential phase was 11.9-fold higher than in stationary phase (25). Taken together, these results highlight the use of P_{cspA} as a tightly controlled sensor for *p*-coumaric acid accumulated in stationary phase.

2.2 Optimization of the fatty acid synthetic pathway

Most cellular malonyl-CoA in wild-type cells is consumed by the fatty acid synthetic pathway which requires an ACP to convert malonyl-CoA to fatty acyl-ACP for extending the saturated fatty acyl chain in each elongation cycles (26). Considering the important role of cofactor protein ACP in fatty acid synthetic pathway, the consumption of malonyl-CoA could be regulated by fine-tuning the activity of ACP. To do so, we overexpressed acyl carrier protein phosphodiesterase (*acpH*) to accelerate the transition from ACP_{active} to $ACP_{inactive}$. (27) Furthermore, the anti-sense RNA of *acpS*, *acpT*, *acpP*, and *fabD* were also overexpressed to repress the translation of ACP, the transition from $ACP_{inactive}$ to ACP_{active} , and the formation of malonyl-ACP (**Fig. 1**). Through the combinatorial regulation of these genes, the concentration of malonyl-CoA was improved to at least 3.61-fold of that of control (**Fig. 3**). Through this analysis, we observed that higher malonyl-CoA concentrations led to lower biomass formation (**Fig. 3**). As a result, a proper dynamic level of *acpH* *asacpS*, and *asacpT* expression is necessary to balance between cell growth and malonyl-CoA accumulation.

Taken together, these results demonstrated that the repression of the transit form $ACP_{inactive}$ to ACP_{active} state is more critical than repressing ACP translation and malonyl-ACP formation (**Fig. 1 and Fig. 3**). This could be explained by the rapid turnover of the AcpS and AcpT catalyzed reaction,

thus resulting in almost exclusive ACP_{active} form (28). Furthermore, overexpression of AcpH not only suppresses the activity of ACP, but could also hydrolyze the 4'-phosphopantetheine moiety of ACP resulting in increasing amounts of CoA and acetyl-CoA after ACP repression (**Supplementary Fig. 2**) (29).

2.3 Designing a (2S)-naringenin-responsive amplifier to balance growth and malonyl-CoA accumulation

In order to dynamically balance cell growth and malonyl-CoA levels, we attempted to construct a (2S)-naringenin-responsive amplifier through coupling the previously optimized (2S)-naringenin synthetic pathway (Module A) (14) with the abovementioned fatty acid repression module (Module B) (**Fig. 4a**). This amplifier could dynamically repress the activity of ACP based on the cellular concentration of (2S)-naringenin. At early growth stages, the slight levels of (2S)-naringenin synthesized by module A will weakly activate module B and thus slowly repress the activity of ACP to produce just enough fatty acid levels to support biomass formation. During cell growth, increasing amounts of (2S)-naringenin will gradually enhance the ACP repression thus further increasing malonyl-CoA levels and (2S)-naringenin productivity. Thus, module B in our scheme is progressively activated to enable high metabolic flux toward (2S)-naringenin (**Fig. 4a**).

To verify this concept, (2S)-naringenin titer and the expression level of *acpH*, *asacpS*, and *asacpT* was determined over the course of fermentation for strains with module A and B. Specifically, we observed a strong positive correlation between fermentation time, (2S)-naringenin concentration and the expression level of *acpH*, *asacpS*, and *asacpT* (**Fig. 4b**). The highest normalized expression levels of *acpH*, *asacpS*, and *asacpT* were 3945, 803, and 4983, respectively, when (2S)-naringenin titer reached 79.2 mg/L (**Fig. 4b**). These results indicate that the constructed amplifier can respond to a broad range of (2S)-naringenin concentration.

To further optimize cell growth, we utilized a three plasmid system with varying copy numbers

to balance module B to enable proper fatty acid biosynthesis and cell growth. In doing so, the (2*S*)-naringenin titer was improved 2.2- to 3.2-fold compared with the control strain only expressing module A (Nar_{modA}) without any impact on cell growth (**Fig. 4c-f**). As a result, the low copy number of module B was selected as the optimal module for (2*S*)-naringenin production. Comparing with the Nar_{modA}, module B-combined strains significantly decreased the accumulation of intermediate *p*-coumaric acid after 14 h fermentation (**Fig. 4c-f**). This phenomenon could be the result of module B function which improved the supply of malonyl-CoA and thus increased the metabolic flux from *p*-coumaric acid-CoA to (2*S*)-naringenin. However, the *p*-coumaric acid of module B-combined strains rapidly accumulated and stop producing (2*S*)-naringenin after cell growth entered stationary phase (**Fig. d-f**). Therefore, the critical challenge left to further improve (2*S*)-naringenin production would be to efficiently utilize the accumulated *p*-coumaric acid in stationary phase.

2.4 Malonyl-CoA synthetic pathway optimization via *p*-coumaric acid-responsive biosensor

Previous study has demonstrated that the concentrations of acetyl-CoA and malonyl-CoA reached the highest levels in exponential phase and rapidly fell to 6.7% and 12.5% of the maximum level when entering stationary phase, respectively (16). Hence, we hypothesize that the accumulation of *p*-coumaric acid and decrease in (2*S*)-naringenin productivity observed in stationary phase was due to the limited availability of malonyl-CoA resulted from the reduced metabolic flux to biosynthesis of acetyl-CoA and malonyl-CoA in the stationary phase. To verify this hypothesis, we measured the concentration of malonyl-CoA of wild type *E. coli* BL21 at different growth phases and confirmed that malonyl-CoA reached its highest level in early growth stage and was almost undetectable in stationary phase (**Fig. 5a**). To enhance the intracellular level of malonyl-CoA, we constructed a dynamically regulated strain, Nar_{modAC} (**Fig. 5b**), through combining the *p*-coumaric acid-responsive dynamic regulation module (module C in **Fig. 1**) with module A. In doing so, the biosynthesis of malonyl-CoA would be enhanced by the overexpression

of *acs*, *ACC*, and the anti-sense RNA of *gltA* (*asgltA*) when *p*-coumaric acid accumulated. In order to optimize this module C, the combinatorial overexpression of *acs*, *ACC*, and *asgltA* was evaluated and we found that overexpression of all genes facilitated the biosynthesis of (2*S*)-naringenin. Among all the constructs, simultaneous overexpression of *acs* and *ACC* leads to the highest (2*S*)-naringenin titer which was 1.5-fold higher than that of Nar_{modA} (**Fig. 5c**).

2.5 Complete DRN assembly for (2*S*)-naringenin production

Based on these collective results, and all modules in place, we combined the optimized modules A, B, and C to construct an artificial DRN which could simultaneously respond with (2*S*)-naringenin and *p*-coumaric acid to enhance the metabolic flux of consumption and biosynthesis of malonyl-CoA, respectively (**Fig. 1 and Fig. 5d**). Our system showed the steady production of *p*-coumaric acid and increasing level of (2*S*)-naringenin in the engineered strain Nar_{modABC} upon entry to stationary phase (**Fig. 5e**). In doing so, the final (2*S*)-naringenin titer reached 4.6-fold (138.9 mg/L) higher than that of Nar_{modA} (30.2 mg/L) and the titer of *p*-coumaric acid stayed nearly constant at around 70 mg/L. These results showcase that module C can effectively utilize *p*-coumaric acid to enhance the biosynthesis of (2*S*)-naringenin. However, the maximum OD₆₀₀ of Nar_{modABC} (6.5) was only 48.1% of that of Nar_{modAB} (13.5) suggesting that the combination of these three modules without fully expression optimization can inhibit the cell growth. Thus, this prompted us to apply a directed evolution strategy to fine-tune the expression level for DRN to rapidly and accurately balance the multiple artificial metabolic systems, and achieve the highest (2*S*)-naringenin titer without impaired cell growth.

2.6 Directed evolution to fine-tune DRN and achieve high level production

To tune the constructed DRN via directed evolution, we established a fluorescent-activated cell sorting (FACS)-based high-throughput screening approach (**Supplementary Fig. 3a**). A TtgR-based

(2*S*)-naringenin biosensor (30) was constructed for FACS screening (**Fig. 6a**) leveraging the detection limit of this sensor (~ 220 mg/L) comparing with other biosensors, FdeR (10 mg/L; **Fig. 2b**) (19, 20) and (2*S*)-naringenin riboswitch (< 100 mg/L) (31, 32). To normalize across cell size and general expression issues, we utilized a constitutively expressing far-red fluorescent protein mKate2 (33) (**Fig. 6a**). As shown in **Fig. 6b**, the TtgR biosensor exhibited a linear correlation between (2*S*)-naringenin concentration and GFP fluorescence when (2*S*)-naringenin was lower than 220 mg/L with a maximum induction of 16.6-fold. Past this concentration, GFP fluorescence decreased and we observed a negative correlation between the FRFP fluorescence and (2*S*)-naringenin concentration likely due to the cell burden of very high expression of GFP. As a result, we sought to identify cells that contain high green fluorescence and low red fluorescence as they are likely candidates for high titer.

To fine-tune the function and performance of our DRN system, the P_{fdeR} - P_{fdeA} of module B and P_{cspA} of module C were randomly mutated and then selected through FACS (**Fig. 6c**). The mutant library sizes were 2.02×10^7 and 1.5×10^6 for module B and C, respectively. After fermentation, the high naringenin-producing strains were sorted (**Supplementary Fig. 3a-c**) and then screened using a 96-well deep well plate assays (14). Among the variants tested, the best performing strain produced 251.4 mg/L (2*S*)-naringenin and 88.3 mg/L *p*-coumaric acid (**Fig. 6d**). When compared with the starting system containing Nar_{modABC} , this mutant was improved 2.7-fold. Moreover, this strain was 1.6-fold improved in OD₆₀₀. In order to characterize mutations in P_{fdeR} - P_{fdeA} and P_{cspA} of derived mutants, the promoter sequences and their relative expression strength were further analyzed (more details in **Supplementary Notes**). Finally, we found that the high expression level of *acpH* is negative to (2*S*)-naringenin production.

3 Discussion

Dynamic control of metabolic pathways marked with precise control of enzyme expression

levels as well as optimal cell growth has received attention in the last few years (34). In this study, we focus on optimizing and developing a NCOMB DRN to dynamically regulate the biosynthesis and conversion of malonyl-CoA in *E. coli* to direct it toward (2S)-naringenin production. In our three-part scheme, the constitutively expressed (2S)-naringenin synthetic pathway (module A) is coupled with a (2S)-naringenin-responsive biosensor (module B) and a stationary-phase malonyl-CoA enhancer (module C) to establish a dynamic amplifier balancing cell growth and malonyl-CoA consumption (**Fig. 1**). It should be noted that in our approach, ACP uniquely serves as the regulatory target instead of the commonly used fatty acid synthase (35). Like most synthetically designed circuits, it was necessary to optimize the interaction parameters in this system using FACS-based directed evolution. The underlying design and principles here have great potential to be extended to produce other valuable target compounds.

As a point of comparison with this work to others in the literature, it is well understood that intracellular malonyl-CoA content and impaired cell growth are the main challenges for (2S)-naringenin production (35). To address these limitations, previous studies have implemented a QS system to enhance malonyl-CoA production after sufficient cell growth. For example, Christina *et al.* established a bifunctional quorum-sensing DRN, which can dynamically enhance the expression level of TAL as well as 4CL and retard malonyl-CoA consumption rate (34). However, the reported (2S)-naringenin titer of best producer (125.9 mg/L) was only 50% of that of Mut-17 generated in this study (251.4 mg/L) (**Fig. 6d**). Comparison with NCOMB DRN, QS-based DRN suffered a signal distortion when transmission from cell density to gene expression. More specifically, we think the signal transition from cell density to malonyl-CoA concentration could not truly reflect the real demands for (2S)-naringenin production since cell growth state is also highly correlated to other factors including the energy and redox homeostasis (ATP/ADP and NADH/NAD⁺), the supply of carbon and nitrogen sources, pH, and temperature (36). In this study, the established DRN could avoid the distortion during signal transmission through directly sensing

the accumulation level of (2*S*)-naringenin and *p*-coumaric acid to dynamically regulate gene expression, and finally achieve the goal of synchronizing cell growth and malonyl-CoA supplementation.

In this study, we successfully demonstrate that the optimized NCOMB DRN allows dynamic regulation of the metabolic fluxes in single cells according to their cell profiles. This strategy resulted in 8.4- and 1.6-fold improvement for (2*S*)-naringenin production and cell growth, respectively, compared to the control strain. We believe that the computational model (deep learning and machine learning modules (37, 38))-based DRN with rational design and optimization can facilitate the improvement in the design accuracy and efficiency for more complex DRNs in the near future.

4 Materials and methods

4.1 Strains, medium, and culture conditions

E. coli JM109 and *E. coli* BL21 were used for plasmids construction and (2*S*)-naringenin production, respectively. LB medium was used to harvest cells for plasmid construction or fluorescence measurement. (2*S*)-naringenin fermentation was performed at 30°C with 220 rpm using MOPS minimal medium (supplemented with 10 g/L D-glucose, 1 g/L peptone, 3 mM L-tyrosine, and 4 g/L NH₄Cl). Streptomycin (100 µg/mL), ampicillin (100 µg/mL), chloramphenicol (34 µg/mL), and kanamycin (50 µg/mL) were added to the media when needed. The strains, plasmids, and primers used in this study are listed in **Supplementary Table 1 and Table 2**.

4.2 Construction and characterization of biosensors

Restriction enzymes were purchased from Thermo Fisher Scientific (Waltham, MA). DNA ligase and PrimeSTAR HS DNA polymerase were purchased from Takara (Dalian, China). A

318 ClonExpress II One Step Cloning Kit (Vazyme Biotech, Nanjing, China) or Gibson assembly
319 method were used for plasmid construction. FdeR and P_{fdeA} promoter were synthesized and
320 codon-optimized by Talen-bio Scientific (Shanghai) Co., Ltd.. Then, FdeR and P_{fdeA} promoter were
321 amplified by primer pairs of FdeR(pACM)-F/FdeA-R, FdeR(pCOM)-F/FdeA-R, and
322 FdeR(pRSM)-F/FdeA-R and cloned into *ApaI/KpnI*, *XbaI/KpnI*, and *XbaI/KpnI* sites of pACM4,
323 pCOM3, and pRSM3 to construct pACM-FdeR, pCOM-FdeR, and pRSM-FdeR, respectively. *gfp*
324 was amplified by primer pairs of GFP-F/GFP-R and cloned into the *SpeI/SalI* sites of plasmid
325 pCDM4 to construct pCDM-GFP. Primer pairs of PfdeA(M)-F/PfdeA(N)-RBS-R or
326 PfdeA(M)-F/PfdeA(1)-R were used to amplify different length of P_{fdeA} (M-N) promoters. Here, M
327 and N represent the positions of amplified promoters, and the last 3' base was defined as position 1.
328 The amplified promoters were cloned into *KpnI/SpeI* sites of pCDM-GFP to construct plasmids of
329 pCDM-PfdeA(M-N)-GFP, *i.e.*, pCDM-PfdeA(180-1)-GFP, pCDM-PfdeA(223-1)-GFP,
330 pCDM-PfdeA(250-1)-GFP, pCDM-PfdeA(288-1)-GFP, pCDM-PfdeA(220-50)-GFP,
331 pCDM-PfdeA(220-83)-GFP, pCDM-PfdeA(220-135)-GFP, and pCDM-PfdeA(220-180)-GFP
332 (**Supplementary Fig. 1a**). To characterize P_{fdeA} (M-N) promoters, the constructed
333 pCDM-PfdeA(M-N)-GFP plasmids were transformed into control strain harboring a pRSM-FdeR
334 plasmid (**Supplementary Fig. 1a**).

335 PadR and its terminator were amplified from the genome of *Bacillus subtilis* 168 by primer
336 pairs of PadR-F/PadR-T-R. pRSM3 was digested by *XbaI/KpnI* to obtain the 1.9 kb and 3.7 kb
337 fragments which were subsequently ligated with PadR-terminator to construct plasmids of
338 pRSM-PadR and pRSM-T₇-PadR, respectively. An exponential phase expressed promoter P_{cspA} was
339 amplified from the genome of *E. coli* MG1655 by primer pairs of cspA-F/cspA-R. Then, P_{cspA} was
340 cloned into *XbaI/AscI* site of pRSM-PadR to construct plasmid pRSM-P_{cspA}-PadR. To simplify the
341 structure of promoter P_{padC}, primer pairs of PadC-P-F/PadC-Q-R were used to amplify different
342 lengths of P_{padC} from the genome of *Bacillus subtilis* 168. Here, P and Q represent the positions of

amplified promoters, and the last 3'-base was defined as position 1. The amplified promoters P_{padC} (P-Q) were ligated with *gfp* by fusion PCR, and then such promoters were cloned into *Bam*HI/*Spe*I sites of pRSM-T7-PadR to construct the plasmids of pRSM-T7-PadR- P_{padC} (P-Q)-GFP (P-Q: 608-1, 608-496, 608-476, 608-456, 216-1, 216-39, 216-79, and 216-123). Furthermore, the P_{padC} -(608-476)-*gfp* fragment was cloned into *Bam*HI/*Spe*I sites of pRSM- P_{cspA} -PadR to construct the plasmids of pRSM- P_{cspA} -PadR- P_{padC} -(608-476)-GFP (Supplementary Fig. 1c).

To characterize the biosensors, strains were cultured in LB medium at 30°C. Various concentrations of (2*S*)-naringenin, IPTG, or/and *p*-coumaric acid were added to induce the expression of GFP when the OD₆₀₀ reached 0.4-0.6. After 8 h induction at 30°C, cells were washed twice in PBS buffer (pH 7.4) and then fluorescence (488 nm excitation, 520 nm emission) and OD₆₀₀ were measured by Cytation 3 imaging reader.

4.3 Pathway construction

Plasmid pUC57-asgltA that carried an anti-sense RNA gene of *gltA* (*asgltA*) was synthesized by Talen-bio Scientific (Shanghai) Co., Ltd.. Promoter P_{fdeA} (223-135) was amplified and cloned into *Not*I site of pUC57-asgltA to construct a pUC57- P_{fdeA} -asgltA plasmid. Furthermore, complementary sequences of mRNA of *acpT*, *acpS*, *acpP*, and *fabD* were amplified from *E. coli* genome by primer pairs of asacpT-F/asacpT-R, asacpS-F/asacpS-R, asacpP-F/asacpP-R, and asfabD-F/asfabD-R, and subsequently cloned into *Nco*I/*Xho*I sites of pUC57- P_{fdeA} -asgltA to construct plasmids of pUC57- P_{fdeA} -asacpT, pUC57- P_{fdeA} -asacpS, pUC57- P_{fdeA} -asacpP, and pUC57- P_{fdeA} -asfabD, respectively. Then, DNA fragments of P_{fdeA} -asacpT, P_{fdeA} -asacpS, P_{fdeA} -asacpP, and P_{fdeA} -asfabD were amplified from these plasmids by primer pairs of asRNA-F/asRNA-R. The *acpH* gene was amplified from the genome of *E. coli* MG1655 by primer pairs of acpH-F/acpH-R, and subsequently cloned into *Kpn*I/*Spe*I sites of pACM-FdeR, pCOM-FdeR, and pRSM-FdeR to construct plasmids of pACM-FdeR-acpH, pCOM-FdeR-acpH, and pRSM-FdeR-acpH, respectively.

368 Finally, P_{fdeA}-asacpT, P_{fdeA}-asacpS, P_{fdeA}-asacpP, and P_{fdeA}-asfabD were digested by *AvrII/SalI*, and
 369 subsequently cloned into *NheI/SalI* sites of pACM-FdeR-acpH, pCOM-FdeR-acpH, and
 370 pRSM-FdeR-acpH by ePathBrick approach (39) to construct module B plasmids of
 371 pACM-FdeR-*acpH*-asacpT, pCOM-FdeR-*acpH*-asacpT, pRSM-FdeR-*acpH*-asacpT,
 372 pACM-FdeR-*acpH*-asacpT-asacpS, pCOM-FdeR-*acpH*-asacpT-asacpS,
 373 pRSM-FdeR-*acpH*-asacpT-asacpS, pACM-FdeR-*acpH*-asacpT-asacpS-asacpP,
 374 pACM-FdeR-*acpH*-asacpT-asacpS-asfabD, and pACM-FdeR-*acpH*-asacpT-asacpS-asacpP-asfabD.
 375 *E. coli* strains that harboring these plasmids were cultured in TB medium and induced by 100 mg/L
 376 (2*S*)-naringenin when OD₆₀₀ reached 0.2. After 2 h induction, cells were harvested to measure the
 377 intracellular concentration of CoA and its esters.

378 Promoter P_{padC} (608-476) was amplified by primer pairs of padC-ACC-F/padC-476-ACC-R
 379 and padC-*SpeI*-F/padC476-acs-R, and subsequently ligated with ACC and acs to construct DNA
 380 fragments of P_{padC}(608-476)-ACC and P_{padC}(608-476)-acs, respectively. Promoter P_{padC} (608-496)
 381 was amplified by primer pairs of padC-*SpeI*-F/padC496-asgltA-R, and subsequently ligated with
 382 asgltA to construct DNA fragments of P_{padC}(608-496)-asgltA. DNA fragments of
 383 P_{padC}(608-496)-asgltA and P_{padC}(608-476)-acs were cloned into *XbaI/NdeI* sites of
 384 pRSM-P_{cspA}-PadR by ePathBrick approach (39) to construct plasmids of pRSM-PadR-acs,
 385 pRSM-PadR-asgltA, and pRSM-PadR-acs-asgltA. Then, P_{padC}(608-476)-ACC was cloned into
 386 *BamHI/SpeI* sites of pRSM-P_{cspA}-PadR, pRSM-PadR-acs, pRSM-PadR-asgltA,
 387 pRSM-PadR-acs-asgltA to construct plasmids of pRSM-PadR-ACC, pRSM-PadR-acs-ACC,
 388 pRSM-PadR-asgltA-ACC, and pRSM-PadR-acs-asgltA-ACC, respectively.

389

390 4.4 FACS-based directed evolution

391 Far-red fluorescent protein (mKate2) and constitutive promoter P₁₁ were amplified from
 392 plasmid pJKR-H-benM (33) with the primer pair of FRFP-F/FRFP-R. (2*S*)-Naringenin-responsive

biosensor (including TtgR, GFP, and promoter P_{TtgR} and P_{TtgABC}) was amplified from pJKR-H-ttgR (30) with the primer pair of TtgR-F/GFP-R. Then the PCR products of mKate2 and (2S)-naringenin biosensor were cloned into *XbaI/BamHI* sites of pCDM- P_{ssrA} -UTR_{tpsT}-CHS-PUTR_{glpD}-CHI by Gibson assembly method to construct the final plasmid pCDM-CHS-CHI-TtgR-GFP-FRFP.

GeneMorph II Random Mutagenesis Kit was used to generate mutation libraries of $P_{\text{fdeR-mut}}$ - $P_{\text{fdeA-mut}}$ and $P_{\text{cspA-mut}}$ with high mutation rate. Then the libraries were Gibson assembled with linearized plasmids of pACM-FdeR-acpH-asacpT-asacpS and RSM-PadR-acs-ACC to construct plasmid libraries of pACM- $P_{\text{fdeR-mut}}$ -FdeR- $P_{\text{fdeA-mut}}$ -acpH-asacpT-asacpS and pRSM- $P_{\text{cspA-mut}}$ -PadR-acs-ACC, respectively. Finally, these libraries were transformed into a *E. coli* strain which carried a constitutively expressing (2S)-naringenin synthetic pathway (module A) to generate a dynamic regulation library of *E. coli* Nar_{lib}.

To perform the flow cytometry sorting, *E. coli* Nar_{lib} was cultured at 30°C and 220 rpm in MOPS medium for 24 h. Then cells were diluted to OD₆₀₀ of 0.5. BD FACSAria III equipped with a 70 µm nozzle, 561 nm and 488 nm laser for excitation, and 610/20 nm and 530/30 nm filter was used for the sorting. The top 0.1% of single cells with high green fluorescence and low red fluorescence were sorted out from the mutant library (approximately 5×10^4). The sorted cells were cultured in LB medium for 8 h at 37°C and then spread on agar plates. Approximately 3000 single colonies were picked into 96-deep-well plates containing 1 mL of MOPS medium in each well to screen high-titer strains by following our previously reported high-throughput screening method. (14) The high producers were further selected and fermented in 250 mL shaking flasks with biological triplicate. Finally, the mutation region of the selected high-titer strains was sequenced.

4.5 Real-time fluorescence quantitative PCR

Strains were cultured in MOPS medium at 30°C with 220 rpm orbital shaking. Cells were then harvested at 4°C with centrifuge speed $3000 \times g$ for 3 min. mRNA was extracted from the harvested

cells according to the manufacturer's instructions of a RNAprep Pure Cell/Bacteria Kit (Tiangen, Beijing, China). PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Japan) was used to reverse transcribe the extracted mRNA. SYBR Premix Ex Taq (Tli RNaseH Plus) was used for qRT-PCR on a LightCycler 480 II thermal cycler system (Roche, Mannheim, Germany). 16S rRNA gene was selected as internal standard.

4.6 Chromatography analysis methods

Equivalent volume of fermentation samples was mixed with ethanol at room temperature to extract (2S)-naringenin and *p*-coumaric acid. Then the mixtures were left at room temperature for 30 min to completely extract products. The supernatant of mixture was collected after centrifugation (4°C, 10000×g, 5 min) and filtered through 0.22 µm hydrophobic membranes. These supernatants were analyzed using an Agilent 1260 HPLC system. (2S)-Naringenin and *p*-coumaric acid were separated by a reverse-phase Gemini NX-C18 column (4.6 × 250 mm) at 25°C. Gradient elution method was the same as described in our previous report (14).

In order to measure the concentration of CoA and its thioester, strains were cultured in TB medium until OD₆₀₀ reached 0.2, and then cells were harvested after induced by 100 mg/L of (2S)-naringenin for 2 h. CoA, acetyl-CoA, and malonyl-CoA were extracted according to the previously described method (40). Shim-pack VP-ODS (250 L ×2.0) UPLC column (Shimadzu, Tokyo, Japan) was used to separate CoA and its thioesters at 40°C with 15 mM ammonium formate in water (A) and 10 mM ammonium acetate in methanol (B) as mobile phases. The elution procedure was used as following: 0.2 mL/min flow rate, 10-25% B (vol/vol) for 5 min, 25-100% B (vol/vol) for 10 min, 100-10% B (vol/vol) for 1 min, maintaining 10% B (vol/vol) for 2 min. For quantitative analysis of CoA and its thioesters, the extracted ion chromatogram (EIC) peak areas were calculated by Shimadu LCMS-IT-TOF (Shimadzu, Kyoto, Japan). The detected EICs were: [M-H]⁻ = 766.5299 *m/z* for CoA, [M+H]⁺ = 810.5813 *m/z* for acetyl-CoA, [M-H]⁻ = 852.5764 *m/z*

443 for malonyl-CoA. All the experiments were performed with biological triplicate. Means are reported,
444 and the error bars represent standard deviation.

445

446 **4.7 Nucleic acid sequences**

447 The nucleic acid sequences of genes, promoters, and anti-sense RNAs were listed in
448 **Supplementary Table 3** of Supplementary materials.

449

450

References:

1. J. J. Wu, G. C. Du, J. W. Zhou, J. Chen, Metabolic engineering of *Escherichia coli* for (2S)-pinocembrin production from glucose by a modular metabolic strategy. *Metab Eng.* **16**, 48-55 (2013).
2. B. Zhang *et al.*, Ribosome binding site libraries and pathway modules for shikimic acid synthesis with *Corynebacterium glutamicum*. *Microb Cell Fact* **14**, 71 (2015).
3. F. A. Ran *et al.*, Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell* **154**, 1380-1389 (2013).
4. X. L. Shen, J. Wang, C. Y. Li, Q. P. Yuan, Y. J. Yan, Dynamic gene expression engineering as a tool in pathway engineering. *Curr Opin Biotech* **59**, 122-129 (2019).
5. F. Moser *et al.*, Dynamic control of endogenous metabolism with combinatorial logic circuits. *Mol. Syst. Biol.* **14**, e8605 (2018).
6. Y. Zheng *et al.*, A tight cold-inducible switch built by coupling thermosensitive transcriptional and proteolytic regulatory parts. *Nucleic Acids Res* **47**, e137 (2019).
7. E. M. Zhao *et al.*, Light-based control of metabolic flux through assembly of synthetic organelles. *Nat Chem Biol* **15**, 589-597 (2019).
8. E. M. Zhao *et al.*, Optogenetic regulation of engineered cellular metabolism for microbial chemical production. *Nature* **555**, 683-687 (2018).
9. B. J. Harder, K. Bettenbrock, S. Klamt, Temperature-dependent dynamic control of the TCA cycle increases volumetric productivity of itaconic acid production by *Escherichia coli*. *Biotechnol Bioeng* **115**, 156-164 (2018).
10. Y. Xiao, C. H. Bowen, D. Liu, F. Zhang, Exploiting nongenetic cell-to-cell variation for enhanced biosynthesis. *Nature chemical biology* **12**, 339 (2016).
11. F. David, J. Nielsen, V. Siewers, Flux control at the malonyl-CoA node through hierarchical dynamic pathway regulation in *Saccharomyces cerevisiae*. *ACS Synth Biol* **5**, 224-233 (2016).
12. P. Xu, L. Y. Li, F. M. Zhang, G. Stephanopoulos, M. Koffas, Improving fatty acids production by engineering dynamic pathway regulation and metabolic control. *P Nat Acad Sci USA* **111**, 11299-11304 (2014).
13. M. L. Skjoedt *et al.*, Engineering prokaryotic transcriptional activators as metabolite biosensors in yeast. *Nat Chem Biol* **12**, 951 (2016).
14. S. Zhou, Y. Lyu, H. Li, M. A. Koffas, J. Zhou, Fine - tuning the (2S)-naringenin synthetic pathway using an iterative high-throughput balancing strategy. *Biotechnol Bioeng* **116**, 1392-1404 (2019).

- 481 15. T. Bretschneider *et al.*, A ketosynthase homolog uses malonyl units to form esters in cervimycin biosynthesis.
482 *Nat Chem Biol* **8**, 154 (2011).
- 483 16. Y. Takamura, G. Nomura, Changes in the intracellular concentration of acetyl-CoA and malonyl-CoA in
484 relation to the carbon and energy metabolism of *Escherichia coli* K12. *J Gen Microbiol* **134**, 2249-2253
485 (1988).
- 486 17. Y. Yang, Y. Lin, L. Li, R. J. Linhardt, Y. Yan, Regulating malonyl-CoA metabolism via synthetic antisense
487 RNAs for enhanced biosynthesis of natural products. *Metab Eng* **29**, 217-226 (2015).
- 488 18. J.-Y. Xu, Y. Xu, X. Chu, M. Tan, B.-C. Ye, Protein acylation affects the artificial biosynthetic pathway for
489 pinosylvin production in engineered *E. coli*. *ACS Chem Biol* **13**, 1200-1208 (2018).
- 490 19. R. Wassem *et al.*, A NodD-like protein activates transcription of genes involved with naringenin degradation in
491 a flavonoid-dependent manner in *Herbaspirillum seropedicae*. *Environ Microbiol* **19**, 1030-1040 (2017).
- 492 20. S. Siedler, S. G. Stahlhut, S. Malla, J. Maury, A. R. Neves, Novel biosensors based on flavonoid-responsive
493 transcriptional regulators introduced into *Escherichia coli*. *Metab Eng* **21**, 2-8 (2014).
- 494 21. S. Siedler *et al.*, Development of a bacterial biosensor for rapid screening of yeast *p*-coumaric acid production.
495 *ACS Synth Biol* **6**, 1860-1869 (2017).
- 496 22. N. P. Tran *et al.*, Phenolic acid-mediated regulation of the *padC* gene, encoding the phenolic acid
497 decarboxylase of *Bacillus subtilis*. *J Bacteriol* **190**, 3213-3224 (2008).
- 498 23. T. K. C. NGUYEN, N. P. TRAN, J.-F. CAVIN, Genetic and biochemical analysis of PadR-*padC* promoter
499 interactions during the phenolic acid stress response (PASR) in *Bacillus subtilis* 168. *J Bacteriol* **196**,
500 4180-4191 (2011).
- 501 24. M. G. Reese, Application of a time-delay neural network to promoter annotation in the *Drosophila*
502 *melanogaster* genome. *Comput Chem* **26**, 51-56 (2001).
- 503 25. S. Zhou *et al.*, Obtaining a panel of cascade promoter-5'-UTR complexes in *Escherichia coli*. *ACS Synth Biol* **6**,
504 1065-1075 (2017).
- 505 26. D. I. Chan, H. J. Vogel, Current understanding of fatty acid biosynthesis and the acyl carrier protein. *Biochem J*
506 **430**, 1-19 (2010).
- 507 27. J. Thomas, J. E. Cronan, The enigmatic acyl carrier protein phosphodiesterase of *Escherichia coli*: genetic and
508 enzymological characterization. *J Biol Chem* **280**, 34675-34683 (2005).
- 509 28. C. S. Heil, S. S. Wehrheim, K. S. Paithankar, M. Grninger, Fatty acid biosynthesis: chain-length regulation
510 and control. *ChemBioChem* **20**, 2298-2321 (2019).

- 511 29. O. C. Sibon, E. Strauss, Coenzyme A: to make it or uptake it? *Nat Rev Mol Cell Bio* **17**, 605-606 (2016).
- 512 30. J. K. Rogers *et al.*, Synthetic biosensors for precise gene control and real-time monitoring of metabolites.
513 *Nucleic Acids Res* **43**, 7648-7660 (2015).
- 514 31. Y. Xiu *et al.*, Naringenin-responsive riboswitch-based fluorescent biosensor module for *Escherichia coli*
515 co-cultures. *Biotechnol Bioeng* **114**, 2235-2244 (2017).
- 516 32. S. Jang *et al.*, Development of artificial riboswitches for monitoring of naringenin in vivo. *ACS Synth Biol* **6**,
517 2077-2085 (2017).
- 518 33. J. K. Rogers, G. M. Church, Genetically encoded sensors enable real-time observation of metabolite
519 production. *Proc Natl Acad Sci USA* **113**, 2388-2393 (2016).
- 520 34. C. V. Dinh, K. L. Prather, Development of an autonomous and bifunctional quorum-sensing circuit for
521 metabolic flux control in engineered *Escherichia coli*. *Proc Natl Acad Sci USA* **116**, 25562-25568 (2019).
- 522 35. J. J. Wu, O. Yu, G. C. Du, J. W. Zhou, J. Chen, Fine-tuning of the fatty acid pathway by synthetic antisense
523 RNA for enhanced (2S)-naringenin production from L-tyrosine in *Escherichia coli*. *Appl Environ Microbiol* **80**,
524 7283-7292 (2014).
- 525 36. J. Shiloach, R. Fass, Growing *E. coli* to high cell density—A historical perspective on method development.
526 *Biotechnol Adv* **23**, 345-357 (2005).
- 527 37. K. M. Chen, E. M. Cofer, J. Zhou, O. G. Troyanskaya, Selene: a PyTorch-based deep learning library for
528 sequence data. *Nat. Methods* **16**, 315-318 (2019).
- 529 38. A. A. Nielsen, C. A. Voigt, Deep learning to predict the lab-of-origin of engineered DNA. *Nat. Commun.* **9**,
530 1-10 (2018).
- 531 39. P. Xu, A. Vansiri, N. Bhan, M. A. G. Koffas, ePathBrick: A synthetic biology platform for engineering
532 metabolic pathways in *E. coli*. *ACS Synth Biol* **1**, 256-266 (2012).
- 533 40. W. J. Zha, S. B. Rubin-Pitel, Z. Y. Shao, H. M. Zhao, Improving cellular malonyl-CoA level in *Escherichia coli*
534 via metabolic engineering. *Metab Eng* **11**, 192-198 (2009).

535

536

Acknowledgements

This work was supported by the National Key R&D Program of China (2019YFA0905502), the National Science Fund for Excellent Young Scholars (21822806), the National Natural Science Foundation of China (31900066, 21877053, 31770097), the Air Force Office of Scientific Research under Award No. FA9550-14-1-0089, the National First-class Discipline Program of Light Industry Technology and Engineering (LITE2018-24), the Fundamental Research Funds for the Central Universities (JUSRP12056, JUSRP51705A).

Author contributions

S. Z., J. Z., and Y. D. conceived the idea. S. Z. performed the experiments and data analysis. S. Z. and S.-F. Y. did the directed evolution. S.-F.Y. and P.H.N. did the 96-well plate screening. H.S.A. suggested the design idea of *p*-coumaric acid biosensor and supported the directed evolution. J. Z., Y. D., and H.S.A. critically reviewed the manuscript. S. Z., S.-F. Y., Y. D., and J. Z. wrote the paper. All authors reviewed, approved and contributed to the final version of the manuscript.

Competing financial interests

The authors declare no competing financial interests.

556 **Figures:**

558 **Figure 1 Schematic of the mechanisms for the NCOMB DRN.**

559 Module A: Heterologous (2S)-naringenin biosynthesis pathway; Module B: Fatty acid repression
560 module. Module A and B could constitute to be a (2S)-naringenin-responsive amplification system
561 to gradually elevate the repression level in malonyl-CoA consumption pathway over the course of
562 the fermentation; Module C: Malonyl-CoA enhancing module. p-Coumaric acid-responsive
563 pathway can dynamically enhance the supply of malonyl-CoA at stationary growth phase. Gene and
564 protein annotations: tyrosine ammonia-lyase (*tal*), 4-coumarate: CoA ligase (*4cl*), chalcone synthase
565 (*chs*), chalcone isomerase (*chi*), citrate synthase (*gltA*), acetyl-CoA synthetase (*acs*), acetyl-CoA
566 carboxylase (ACC), acyl carrier protein (ACP), flavonoids-responsive transcriptional activator from
567 *Herbaspirillum seropedicae* SmR1 (FdeR), p-coumaric acid-responsive transcriptional repressor
568 from *Bacillus subtilis* (PadR), acyl carrier protein phosphodiesterase (*acpH*). *asfabD*, *asacpS*,
569 *asacpT*, *asacpP*, and *asgltA* are anti-sense RNAs. Green arrows: constitutive expression pathway.

Blue dash arrows: activation reactions; Blue dash blunt arrows: repression reactions. Bold arrows: enhanced metabolic fluxes.

Figure 2 The structures and properties of (2S)-naringenin- and p-coumaric acid-responsive biosensors.

a: The original structure of P_{fdeA} bidirectional promoter and truncated P_{fdeA} (M-N) regions. Red bar represents FdeR binding site (T-N11-A). **b:** The structure of (2S)-naringenin-responsive biosensor. **c:** The expression strength of promoter P_{fdeA} (M-N) when induced by various concentrations of (2S)-naringenin. Control represents strain only harboring pRSM-FdeR. Strains labeled with M-N represent constructs carrying pCDM-PfdeA(M-N)-GFP and pRSM-FdeR (Supplementary Fig. 1a).

d: The predicted -35/-10 boxes of P_{padC} and truncated P_{padC} (P-Q) regions. **e:** The structure of IPTG- and *p*-coumaric acid responsive biosensor. **f:** The expression strength of promoter P_{padC} (P-Q). **g:** The structure of *p*-coumaric acid-responsive biosensor. **h:** The properties of IPTG and/or *p*-coumaric acid inducible biosensors. Error bars represent standard error of biological triplicates.

Figure 3 Fatty acid pathway optimization by (2S)-naringenin-responsive biosensor.

Genes were overexpressed in pACM-FdeR plasmid under the control of P_{fdeA} (223-135) with 100 mg/L (2S)-naringenin induction. + or - represent the related genes were overexpressed or not, respectively. The control strain only harboring pACM-FdeR. Error bars represent standard error of biological triplicates.

Figure 4 The mechanisms and properties of (2S)-naringenin-responsive amplifier.

a: The mechanisms of (2S)-naringenin-responsive amplifier. **b:** The relative mRNA level and (2S)-naringenin titer of module A and B combined strain at different fermentation time. pACM-FdeR-*acpH-asacpT-asacpS* was used as module B. Blue, green, and orange points represent the genes of *asacpT*, *asacpS*, and *acpH*, respectively. **c-f:** The fermentation of different module A and B combined strains. The control strain (Nar_{modA}) only harbors module A (c). Module A combined with pACM-FdeR-*acpH-asacpT-asacpS* (low copy number), pCOM-FdeR-*acpH-asacpT-asacpS* (medium copy number), pRSM-FdeR-*acpH-asacpT-asacpS* (high copy number) to generate strains of $Nar_{modAB-L}$ (d), $Nar_{modAB-M}$ (e), and $Nar_{modAB-H}$ (f), respectively. Error bars represent standard error of biological triplicates.

Figure 5 The mechanisms and properties of *p*-coumaric acid-responsive dynamic regulation strains.

a: The malonyl-CoA concentration of wild type *E. coli* BL21 in different growth stages. **b:** The regulation mechanisms of strain Nar_{modAC}. **c:** Optimization of module C through the combinatorial gene overexpression to generate various Nar_{modAC} constructs. Nar_{modA} was used as control. + or - represent the related genes were overexpressed or not, respectively. **d:** The regulation mechanisms of strain Nar_{modABC}. Module B and C were pACM-FdeR-*acpH*-*asacpT*-*asacpS* and pRSM-PadR-*acs*-*ACC*, respectively. **e:** The (2S)-naringenin, *p*-coumaric acid, and OD₆₀₀ level of Nar_{modABC} were determined. Error bars represent standard error of biological triplicates.

Figure 6 The FACS-based directed evolution.

a: The structure of TtgR based (2S)-naringenin-responsive biosensor. **b:** The fluorescence levels of TtgR biosensor with different concentration of (2S)-naringenin inducer. **c:** The structure of module B and C. Red region represent the mutation area. **d:** The (2S)-naringenin, *p*-coumaric acid, and OD_{600} level of screened high titer strains. Error bars represent standard error of biological triplicates.