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Fine-tuning the (2S)-naringenin synthetic pathway using an iterative high-throughput balancing strategy

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Abstract
Metabolic engineering consistently demands to produce the maximum carbon and energy flux to target chemicals. To balance metabolic flux, gene expression levels of artificially synthesized pathways usually fine-tuned using multimodular optimization strategy. However, forward construction is an engineering conundrum because a vast number of possible pathway combinations need to be constructed and analyzed. Here, an iterative high-throughput balancing (IHTB) strategy was established to thoroughly fine-tune the (2S)-naringenin biosynthetic pathway. A series of gradient constitutive promoters from Escherichia coli were randomly cloned upstream of pathway genes, and the resulting library was screened using an ultraviolet spectrophotometry–fluorescence spectrophotometry high-throughput method, which was established based on the interactions between AlCl₃ and (2S)-naringenin. The metabolic flux of the screened high-titer strains was analyzed and iterative rounds of screening were performed based on the analysis results. After several rounds, the metabolic flux of the (2S)-naringenin synthetic pathway was balanced, reaching a final titer of 191.9 mg/L with 29.2 mg/L p-coumaric acid accumulation. Chalcone synthase was speculated to be the rate-limiting enzyme because its expression level was closely related to the production of both (2S)-naringenin and p-coumaric acid. The established IHTB strategy can be used to efficiently balance multigene pathways, which will accelerate the development of efficient recombinant strains.

KEYWORDS
flavonoids, metabolic engineering, modular optimization, promoter
1 | INTRODUCTION

Synthetic biology has created diverse cell factories to produce many useful chemicals (Becker & Wittmann, 2016; Dai & Nielsen, 2015; Vuoristo, Mars, Sanders, Eggink, & Weusthuis, 2016). With the development of systems biology and next-generation sequencing, more key enzymes from plants and microbes have been mined to assemble recombinant pathways in different hosts (Tatsis & O’Connor, 2016; P. Xu, Bhan, & Koffas, 2013). Often times, such heterologous enzymes exhibited diverse activities in different hosts, especially when eukaryotic enzymes are expressed in prokaryotic cells (Jendresen et al., 2015; Santos, Koffas, & Stephanopoulos, 2011; Zhu, Wu, Du, Zhou, & Chen, 2014). Overexpressing all of the high-activity enzymes in a pathway can waste energy and precursors, resulting in the engineered strains’ decreased performance (Kittleson, Wu, & Anderson, 2012; G. Wu et al., 2016). Thus, a crucial challenge in metabolic engineering is balancing the metabolic flux to increase product titer, decrease intermediate metabolite accumulation, and improve cell growth (Biggs, De Paepe, Santos, De Mey, & Ajikumar, 2014; Dueber et al., 2009; Jones, Toparliak, & Koffas, 2015).

To improve the titer of target chemicals, researchers first focused on screening for high-activity enzymes in plants, animals, fungi, and bacteria (Jendresen et al., 2015; Santos et al., 2011). If the screened enzymes cannot satisfy present requirements of metabolic engineering, then protein engineering strategies, like directed evolution (S. Zhou & Alper, 2018; S. Zhou et al., 2016) and computer-aided methods (Ebert & Pelletier, 2017; Fang, Zhang, Du, & Chen, 2017; Verma, Schwaneberg, & Roccatano, 2012), are typically performed to further improve the properties of the target enzymes. However, high-efficiency biosynthetic pathways are through the use and regulated expression of high-activity enzymes. To balance the metabolic flux, a series of computational-based pathway design strategies (Long, Ong, & Reed, 2015) have been established and successfully used for the production of desired compounds, such as flavonoids (P. Xu, Ranganathan, Fowler, Maranas, & Koffas, 2011), 1,4-butanediol (Yim et al., 2011), and succinate (Ranganathan, Suthers, & Maranas, 2010). However, due to the lack of accurate parameters for enzyme reactions, the use of these regulatory strategies is usually restricted.

Recently, modular optimization strategies, based on promoters with different strengths and plasmids with different copy numbers, have increased the titers of several chemicals, such as terpenes (Ajikumar et al., 2010; Y. Z. Zhou et al., 2012), flavonoids (J. Wu, Du, Zhou, & Chen, 2013), amino acids (Qin et al., 2015), and fatty acids (P. Xu, Gu et al., 2013). However, such optimization approaches are often limited by a lack of genetic tools, such as promoters and plasmids. More specifically, a key element in balancing metabolic fluxes is the availability of promoters with gradient strengths (S. Zhou, Ding et al., 2017; S. Zhou, Du et al., 2017). In addition, testing such promoters in multigene pathways results in an astronomical number of combinations. Thus, construction and screening of all possible gene–promoter combinations by high-throughput methods become a promising strategy to thoroughly fine-tuning multigene pathways.

(2S)-Naringenin is an early compound in flavonoid biosynthesis that has a broad range of nutritional and pharmaceutical properties (Manchope, Casagrande, & Verri, 2017). Even though significant advances have been made to efficiently synthesize (2S)-naringenin in recombinant microorganisms (J. Zhou, Du, & Chen, 2014), several challenges still exist. For example, at least six heterologous genes need to be expressed and balanced in Escherichia coli or Saccharomyces cerevisiae (Figure 1). In this study, we established an iterative high-throughput balancing (IHTB) strategy to fine-tune the (2S)-naringenin synthetic pathway in E. coli. Based on the reaction theory between metal ion and flavonoids (Kaspzrak, Erxleben, & Ochocki, 2015), AlCl₃ was used as an auxochrome for establishing a high-throughput screening method. Pathway libraries were established by randomly introducing promoters with gradient strengths (S. Zhou, Ding et al., 2017) upstream of the biosynthetic genes (Figure 2). After several rounds of construction, screening, and analysis, (2S)-naringenin production reached 191.9 mg/L with 29.2 mg/L p-coumaric acid accumulation. The titer of (2S)-naringenin is twice that of our previous report (J. Wu, Zhou, Du, Zhou, & Chen, 2014), which used a modular optimization strategy to balance the pathway. The IHTB strategy described in this study as a novel metabolic flux balancing strategy has enormous potential to efficiently fine-tune other multigene pathways.

2 | MATERIALS AND METHODS

2.1 | Reagents, medium, and strains

Standard samples of (2S)-naringenin (S530098-sample), p-coumaric acid (55823-50 mg) and l-tyrosine (T8566–25 g) were purchased...
Constitutive gradient promoters from *E. coli* were amplified and mixed at equimolar final concentrations to prepare suitable promoter libraries. Appropriate promoter libraries were randomly inserted upstream of target genes, resulting in two plasmid libraries, pET-γ-TAL-α-4CL and pCDF-δ-CHS-β-CHI. Intermediate metabolite concentrations and promoters of the screened high-titer strains were analyzed for the next round of screening. Red wells in the 96-well plates represent high-titer strains. CHI: chalcone isomerase; CHS: chalcone synthase; TAL: tyrosine ammonia-lyase; 4CL: 4-coumarate-CoA ligase.

[Color figure can be viewed at wileyonlinelibrary.com]
2.2 | Promoter library preparation

Based on our previous study (J. Wu, Zhou et al., 2014), the optimal expression ratio of (2S)-naringenin biosynthetic genes are Tctal:Pe4cl: Phchs:Mschi = 1:1:10:10. Hence, in the first round of screening, 16 promoters (PUTRrpsT, PUTRrpsL, PUTRrpsU, PUTRrrnA, PUTRglpD, PUTRssrS, PUTRaccBC, PUTRalsRBACE, PUTRrpsT, PUTRrplNXE, PUTRrplNL, PUTRgrpE, PUTRalsRBACE, PUTRrrnG) were used to regulate the expression of CHS genes. Each of the promoter activity levels is listed in Table S3. Two sets of plasmid libraries were randomly cloned into the pETM6 plasmid, respectively, resulting in plasmid pETM6 library 1 (J. Wu, Zhou et al., 2014). The optimal promoters were used to synthesize 30 single colonies from each of the pET libraries, pETM6CHS, pETM6CHI using the One Step Cloning Kit, resulting in two plasmid libraries, pETM6-CHS and pETM6-CHI (Figure 2). Greek letters indicate the potential inserted PUTR sequences. A total of 192 single colonies from each of the plasmid libraries was incubated in 96-deep-well plates using the QPiX420 system (Molecular Devices, Sunnyvale, CA).

After overnight culturing, 0.7 ml of each of the 192 strains carrying plasmids pET-TAL-α-4CL or pCDF-CHS-β-CHI were mixed. Then, the plasmid libraries of pET-TAL-α-4CL and pCDF-CHS-β-CHI were extracted from the mixed culture broth. Subsequently, promoters were randomly cloned into BamHI/HindIII sites of pET-TAL-α-4CL or pCDF-CHS-β-CHI using the One Step Cloning Kit, resulting in two plasmid libraries, pET-γ-TAL-α-4CL and pCDF-δ-CHS-β-CHI (Figure 2).

2.3 | Plasmid library construction

The Tctal (Jendresen et al., 2015) was synthesized and codon-optimized by GenScript (Nanjing, China). The previously codon-optimized genes, Pe4cl, Phchs, and Mschi, were used for (2S)-naringenin production (J. Wu, Zhou et al., 2014). The Tctal and Pe4cl genes were amplified and cloned into the AvrII/Xhol and Xhol/SpeI sites of the pETM6 plasmid, respectively, resulting in plasmid pETM-3-4CL. The Phchs and Mschi genes were amplified and cloned into the AvrII/Xhol and Xhol/SpeI sites of plasmid pHCM4, respectively, resulting in plasmid pHCM-CHS-4CL. A strong PUTR complex P PUTR rpsT (S. Zhou, Ding et al., 2017) was cloned by primer pairs ABCg1/F-ABCg2/R and ABCg2/F-ABCg2/R and inserted into the EcoRI/Apol and Nott/Ndel sites, respectively, of the pACYC-matC-matB plasmid (Leonard et al., 2008). The resulting plasmid pACYC-matC-matB (P PUTR rpsT) increased the intracellular concentration of malonyl-CoA. T7 promoter was amplified by primer pairs of T7-Gib-BamHI/F-T7-Gib-HindIII-R from pCDF4uet1 plasmid. Then the amplified T7 promoter was cloned into BamHI/HindIII site of pCDM-P PUTR rpsT-CHS-P PUTR CHI of 9G3 strain by Gibson assembly and resulted in a new plasmid pCDM-T7-CHS-P PUTR CHI. All of the primers used in this study are listed in Table S4, while plasmids and strains are listed in Table 1.

Promoters were amplified from the genome of E. coli K12 MG1655. The amplified promoters flanked their cloning sites with 15nt homologous sequences. Then, promoters were mixed at an equivalent molar ratio (0.06 pmol) and randomly inserted independently into NotI/Smal sites of pETM-TcTAL-4CL and pCDM-CHS-CHI using a One Step Cloning Kit, resulting in two plasmid libraries, pET-TAL-α-4CL and pCDF-CHS-β-CHI (Figure 2). Greek letters indicate the potential inserted PUTR sequences. A total of 192 single colonies from each of the plasmid libraries was incubated in 96-deep-well plates using the QPiX420 system (Molecular Devices, Sunnyvale, CA).

After overnight culturing, 0.7 ml of each of the 192 strains carrying plasmids pET-TAL-α-4CL or pCDF-CHS-β-CHI were mixed. Then, the plasmid libraries of pET-TAL-α-4CL and pCDF-CHS-β-CHI were extracted from the mixed culture broth. Subsequently, promoters were randomly cloned into BamHI/HindIII sites of pET-TAL-α-4CL or pCDF-CHS-β-CHI using the One Step Cloning Kit, resulting in two plasmid libraries, pET-γ-TAL-α-4CL and pCDF-δ-CHS-β-CHI (Figure 2). A total of 1,920 single colonies from pET-TAL-α-4CL and pCDF-δ-CHS-β-CHI were incubated into 96-deep-well plates using the QPiX420 system. After overnight culturing, 0.7 ml of each of the 1,920 strains containing pET-γ-TAL-α-4CL or pCDF-δ-CHS-β-CHI were mixed. Then, the plasmid libraries of pET-γ-TAL-α-4CL and pCDF-δ-CHS-β-CHI from the mixed culture broth were extracted. To detect the diversity in library construction, we randomly picked and sequenced 30 single colonies from each of the pET-TAL-α-4CL and pCDF-CHS-β-CHI libraries.

2.4 | Spectrophotometry analysis in 96-well plates

Metal ions can bind the 5-hydroxyl and 4-carbonyl groups of flavonoids and generate a complex compound, which can display a fluorescence emission (Kasprzk et al., 2015; Selvaraj, Krishnaswamy, Devashya, Sethuraman, & Krishnan, 2014). While, H2BO3 bind some of flavonoids generate yellow products (Jurd, 1956). To shift the maximum absorption peak (MAP) of (2S)-naringenin, AlCl3, MgOAc, and H2BO3 were used. Cultured MOPS minimal medium was used to dissolve AlCl3, MgOAc, and H2BO3 to a final 20 g/L concentration to simulate the culturing process. (2S)-Naringenin, p-coumaric acid, and l-tyrosine were dissolved in dimethyl sulfoxide. Equivalent volumes of 2% AlCl3, 2% MgOAc, or 2% H2BO3 were mixed with 400 mg/L (2S)-naringenin, 80 mg/L p-coumaric acid, or 100 mg/L l-tyrosine. The emission and excitation wavelengths of the
mixtures in standard 96-well fluorescent plates (Corning) were scanned. The obtained maximum excitation and maximum emission wavelengths were chosen as detecting wavelengths for (2S)-naringenin, p-coumaric acid, and l-tyrosine analyses.

2.5 Optimization and verification of the detection methods

(2S)-Naringenin can be analyzed by FS and UVS. To optimize the FS testing conditions, the cultured MOPS medium was used as the solvent to prepare 1%, 2%, 4%, and 6% of AlCl₃ and MgOAc. Dimethyl sulfoxide was used to prepare 10, 20, 30, 40, and 50 mg/L of the (2S)-naringenin standard solution. Equivalent volumes of 1% AlCl₃ were mixed with 10, 20, 30, 40, and 50 mg/L of the (2S)-naringenin standard solution to prepare mixtures with final (2S)-naringenin concentrations of 5, 10, 15, 20, and 25 mg/L. Their fluorescence emissions were then detected at 382 nm excitation and 505 nm emission. Based on the fluorescence strength, the standard curve of (2S)-naringenin was established (Table S1). Using the same procedure, the other standard curves of (2S)-naringenin were established for different metal ion concentrations in MOPS medium according to the conditions listed in Table S1. The R² square values were used to evaluate the optimum metal ion concentrations. To establish a (2S)-naringenin UVS assay procedure, the standard curve of (2S)-naringenin, at concentrations of 30, 60, 90, 120, 150, 180, 210, 240, 270, and 300 mg/L, was measured at 373 nm in a 1% AlCl₃ solution.

Cross-experiments were performed to verify whether the coexistence of p-coumaric acid, l-tyrosine, and (2S)-naringenin could interrupt the quantitative analysis procedure. In these cross-experiments, the reaction systems were prepared by mixing equivalent volumes of cultured MOPS medium containing different metal ions and different concentrations of (2S)-naringenin, p-coumaric acid, and l-tyrosine. The concentration of (2S)-naringenin in each solution was detected by both UVS-FS and high performance liquid chromatography (HPLC) methods. Details of the cross-experiment reaction systems are listed in Table 2.

2.6 High-throughput screening strategy

The plasmid libraries of pET-γ-TAL-α-4CL and pCDF-δ-JSH-β-CHI were cotransformed into E. coli MalCoA competent cells, resulting in a
library of (2S)-naringenin production strains (Figure 2). To screen for high-titer strains, single colonies were incubated in 96-deep-well plates with 500 µl of MOPS medium supplemented with 3 mM l-tyrosine and 2 g/L sodium malonate. After 48 hr of culturing at 30°C and 220 rpm, 96-deep-well plates were centrifuged at 4,000×g for 10 min to separate cells and medium. Then, 100 µl of supernatant was mixed with the equivalent volume of 4% AlCl₃ solution in 96-well fluorescence plates, and the fluorescence level and UV absorbance were measured at 382 nm excitation, 505 nm emission, and 373 nm using the TECAN Freedom EVO Automatic Workstation (Tecan, Männedorf, Switzerland). The strains having the highest fluorescence level and the highest UV absorbance were selected as high-titer candidate strains. Four high-titer candidate strains were selected from each of the 96-deep-well plates for secondary screening in flasks.

For the flasks screening, the high-titer candidate strains were cultured in 50-ml conical flasks with 5 ml MOPS medium at 30°C and 220 rpm orbital shaking for (2S)-naringenin production. The fermentation process was terminated after 72 hr (first round screening) or 48 hr (second round screening) of culturing. Then the HPLC was used to accurately measure the titer of (2S)-naringenin and p-coumaric acid. After that, strains with gradient titers were selected, and the promoters that were located upstream of the genes Tctal, Pc4cl, Phchs, and Mschi were sequenced. Promoter strength reflects the expression level of downstream genes. By comparing the titer, intermediate metabolism, and gene expression levels, the rate-limiting step and metabolic flux were analyzed. In the next round of high-throughput screening, the range of promoters used in the library was reduced to further improve the expression level of the rate-limiting step. The fermentation process of control strain S5 is followed by our previous reports (J. Wu, Zhou et al., 2014). The flowchart of the IHTB strategy is illustrated in Figure 2.

### 2.7 HPLC analysis

To quantify the levels of (2S)-naringenin, 1-ml samples were dried by evaporation at ~50°C with a 100°C motor vacuum using VirTis Freeze Drying Equipment (SP Scientific, Warminster, PA), and the resulting residue was redissolved in 1 ml of methanol. These redissolved samples were analyzed using an Agilent 1260 HPLC system. A reverse-phase Gemini NX-C18 column (4.6 × 250 mm) was used for the purification and separation of (2S)-naringenin and p-coumaric acid at 25°C. Gradient elution was used to separate (2S)-naringenin and p-coumaric acid using the following protocol: 1.0 ml/min flow rate, 10 to 40% acetonitrile (vol/vol) for 10 min, 40% acetonitrile (vol/vol) for 5 min, and 40 to 10% acetonitrile (vol/vol) for 2 min (J. Wu, Zhou et al., 2014).

### 3 RESULTS

#### 3.1 Spectral characteristics of (2S)-naringenin

Due to the presence of carbonyl and hydroxyl groups in flavonoids, spectral properties of some flavonoids could be altered by some specific metal ions (Kasprzak et al., 2015). The altered spectral properties could then be applied for the quantitative measurement of some flavonoids (Pyrzynska & Pekal, 2011). In the previous study, aluminum was usually used to measure total flavonoids of plant extractions (Papoti, Xystouris, Papagianni, & Tsimidou, 2011). Since the detection environment could influence the reaction between the metal ion and flavonoids (Jurd & Geissman, 1956), there have no reports for quantitative detection of flavonoids in culture broth using metal ion complex based method.

A comparison of the UV-visible absorption spectra of (2S)-naringenin, p-coumaric acid, and l-tyrosine showed that their absorption peaks overlap (Figure 3a). Thus, it is difficult to measure the (2S)-naringenin content directly in a culture broth when the other chemicals are present. A new absorption peak at 373 nm was observed in the AlCl₃ solution of (2S)-naringenin (Figure 3b). It is possible that (2S)-naringenin can be measured in the AlCl₃ solution using ultraviolet spectrophotometry (UVS) at 373 nm. Furthermore, no significant influence on the (2S)-naringenin UV-visible absorption spectrum was observed when MgOAc and H₂BO₃ were used to shift the (2S)-naringenin MAP in the MOPS medium (Figure 3c,d). No fluorescent signal was obtained when (2S)-naringenin, p-coumaric acid, and l-tyrosine were dissolved in cultured MOPS medium without the addition of a metal ion (Figure 3e,f). Fluorescence scanning showed that the maximum emission wavelengths of (2S)-naringenin in 1% AlCl₃ and 1% MgOAc were 505 and 492 nm, respectively (Figure 3g,h), whereas the maximum excitation wavelengths were 382 and 371 nm, respectively (Figure 3i,j). The high fluorescence strength, low background, and intermediate metabolite interference in AlCl₃ and MgOAc solutions makes the FS analysis of (2S)-naringenin possible.

#### 3.2 Optimized screening of (2S)-naringenin

The standard curves of a series of reaction systems were established for optimizing metal ion concentrations, which may influence the analysis of

### Table 2: Cross-over experiment reaction systems

<table>
<thead>
<tr>
<th>2% AlCl₃⁺⁺ (mg/L)</th>
<th>1% MgOAc⁺⁺ (mg/L)</th>
<th>2% AlCl₃⁺⁺ (mg/L)</th>
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<td>9/10/50</td>
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<td>30/70/50</td>
<td>210/240/50</td>
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*The numbers represent concentrations of (2S)-naringenin/p-coumaric acid/l-tyrosine in every cross-experiment.
*The cross-experiments were performed to evaluate whether intermediate metabolites have an effect on (2S)-naringenin fluorescence spectrophotometry assay procedure.
*The cross-experiments were performed to evaluate whether intermediate metabolites have an effect on (2S)-naringenin fluorescence spectrophotometry assay procedure.
*The (2S)-naringenin ultraviolet spectrophotometry assay procedure was analyzed to investigate the intermediate metabolite interruption at 373 nm.
(2S)-naringenin. According to the \( R^2 \) values, the concentration of \( Al^{3+} \) and \( Mg^{2+} \) from 0.5% to 3% have no significant influence on (2S)-naringenin detection (Table S1). Finally, the optimum conditions for a (2S)-naringenin FS analysis were selected as 2% AlCl\(_3\) \( (R^2 = 0.9994) \) and 1% MgOAc \( (R^2 = 0.9999) \) solution. To further verify the reliability of the UVS–FS measurements, cross-experiments were performed. By comparing HPLC and the UVS–FS testing concentration, the optimum conditions for the (2S)-naringenin analysis were determined (Figure 3k,l). The FS analysis of (2S)-naringenin in a 2% MgOAc solution (Figure 3m) was consistent with the commonly used HPLC method.
3.3 | Plasmid library evaluation

Sixteen gradient strength PUTRs (promoter-untranslated region) were randomly cloned upstream of Tctal and Pc4cl (Tctal encoding for tyrosine ammonia-lyase from Trichosporon cutaneum, Pc4cl encoding for 4-coumarate-CoA ligase from Petroselinum crispum) to generate a library with 256 theoretic diversity. Likewise, another 15 gradient strength PUTRs were randomly cloned upstream of Phchs and Mschi (Phchs encoding for chalcone synthase from Petunia X hybrid, Mschi encoding for chalcone isomerase from Medicago sativa) to generate another library with 225 theoretic diversity. Then the two libraries were cotransformed into E. coli BL21(DE3) and resulted in a final (2S)-naringenin library with theoretic diversity of 57,600 strains (Table S2).

Efficiency and diversity are two crucial criteria that directly determine the success of plasmid libraries. To evaluate the efficiency of plasmid libraries, the number of single colonies obtained from the negative controls (Figure 4a). In addition, colony PCR was conducted to evaluate the ratio of false positives (Figure 4b). Almost no false positive strains appeared, and the plasmid libraries had high construction efficiency levels (Figure 4b). Due to the promoters used for library construction have different length, the various size of PCR bands on the gel revealed each library have acceptable diversity. To evaluate the library diversity, 30 strains each from the pET-TAL-α-4CL, pCDF-CHS-β-CHI, pET-γ-α-4CL, and pCDF-δ-CHS-β-CHI libraries were randomly selected and sequenced, respectively. More than 60% of the promoters appeared in the 30 sequenced strains (Table S2). The limited number of sequenced samples may explain why a large percentage of the existing promoters were not found.

3.4 | First round of high-throughput screening

After screening 4,800 strains in 96-deep-well plates, 200 high-titer strains were selected for further fermentation in 50-mL conical flasks. Due to their different growth rates, fermentation was terminated with the same OD600 after culturing for 72 hr. Then the highest (2S)-naringenin production strain was screened with 124.7 mg/L titer (Figure 5), whereas p-coumaric acid accumulation reached 723.3 mg/L (Figure 5). To analyze the metabolic flux, 25 strains with gradient titers were selected and their promoters were sequenced. Based on the reported promoter strengths (S. Zhou, Ding et al., 2017), the expression levels of the Tctal, Pc4cl, Phchs, and Mschi genes were quantified (Figure 5). The results showed that: (a) high expression levels of Phchs promoted the production of (2S)-naringenin; (b) both Pc4cl and Mschi have no obvious impact on (2S)-naringenin production; (c) a comparatively lower expression level of Tctal contributed to (2S)-naringenin

**FIGURE 4** Evaluation of plasmid library efficiencies A: a–d, Many colonies on the plates show high ligation and transformation efficiencies in the pET-TAL-α-4CL (a), pCDF-CHS-β-CHI (b), pET-γ-α-4CL (c), and pCDF-δ-CHS-β-CHI (d) plasmid libraries. e–h, Limited colonies on plates of the negative controls for pET-TAL-α-4CL (e), pCDF-CHS-β-CHI (f), pET-γ-α-4CL (g) and pCDF-δ-CHS-β-CHI (h). B: Agar gel electrophoresis of colony PCR from pET-TAL-α-4CL, pCDF-CHS-β-CHI, pET-γ-α-4CL and pCDF-δ-CHS-β-CHI libraries. M: Marker lane (DL 2000; TaKaRa, Dalian, China)

**FIGURE 5** Effects of gene expression levels on the accumulation of (2S)-naringenin and p-coumaric acid in the first round of screening. The expression level of the PBAD promoter after induction with 10 mM arabinose was defined as 1. By comparing with the PBAD promoter’s expression, the expression levels of the Tctal, Pc4cl, Phchs, and Mschi genes were standardized and accurately quantified [Color figure can be viewed at wileyonlinelibrary.com]
production. Since the instability of other intermediates, such as coumaroyl-CoA and naringenin-chalcone, lead to difficulties in obtaining of standards and preparation of samples to achieve the reliable quantitation, only the accumulation of p-coumaric acid was measured here. Therefore, to further improve the production of (2S)-naringenin and decrease the accumulation of p-coumaric acid, expression levels of Phchs should be enhanced.

3.5 Iteration high-throughput screening and rate-limiting step analysis

In the second round of high-throughput screening, the promoter library used for regulating the Tctal expression was the same one used in the first round of screening. Three strong promoters (PUTRssrA, PUTRinfC, and PUTRinfC-PUTRintC-rpsT) (PUTR, promoter and 5′-untranslated region complex), a medium strength promoter (PUTRrpsT), and 2 weak promoters (PUTRpsT and PUTRtpst) were used to regulate the expression of Pc4cl. Three strong promoters (PUTRssrA-PUTRintC-rpsT, PUTRrpsT, and PssrA-UTRtpst), and a medium strength promoter (PUTRtpst) were used to regulate the expression levels of Phchs and Mschi.

After screening 1,200 strains, a high-titer strain (9G3) that produced 191.9 mg/L (2S)-naringenin and accumulated 29.2 mg/L p-coumaric acid was identified (Figure 6). No more efficient producing strains were identified in subsequent rounds of screening. The identified strain, 9G3, carried the same expression vectors and pathway genes as strain S5 identified in our previous report (J. Wu, Zhou et al., 2014); however, strain S5 used isopropyl-β-d-thiogalactoside induced promoters to drive gene expression. The (2S)-naringenin and p-coumaric acid production levels of strain 9G3 are 2.1- and 0.3-fold of those of strain S5, respectively. This indicates that, compared with the limited number of inducible promoters, the large number of constitutive promoters are more powerful for fine-tuning multigene pathways. To analyze the rate-limiting step of (2S)-naringenin production, the promoters of 12 high-titer strains were identified (Figure 6). By increasing the CHS expression level, (2S)-naringenin production increased from 80.3 to 135.7 mg/L and the accumulation of p-coumaric acid decreased from 104.5 to 20.7 mg/L (4D9, 1D4, 10A10, 10F9, and 8H1 strains). However, regulating the TAL, 4CL, and CHI expression levels had little effect on (2S)-naringenin production (Figure 6). Thus, CHS is most likely the rate-limiting enzyme in (2S)-naringenin production.

To further verify this speculation, the upstream promoter of CHS in 9G3 strain was replaced by a T7 promoter and resulted in a strain of E. coli BL21 9G3-CHST7. After 48 h fermentation, the (2S)-naringenin titer gradually improved with the improvement of isopropyl-β-d-thiogalactoside (IPTG) concentration from 0 to 0.1 mM, whereas p-coumaric acid titer gradually decreased (Figure 7). However, too strong expression of CHS may lead to inclusion body generation, low cell final OD and further resulted low (2S)-naringenin production and p-coumaric acid accumulation.

4 DISCUSSION

Metabolic engineering strives to achieve maximum energy and substance flux for the biosynthesis of targeted recombinant chemicals without negatively affecting cell growth due to the depletion of resources, a
phenomenon commonly known as metabolic burden (G. Wu et al., 2016). Thus, balancing multigene pathways is of utmost importance and has already been applied to the biosynthesis of recombinant chemicals, such as violacein from glucose, using a T7 promoter library (Jones et al., 2015), and flavanones from methanol libraries (Whitaker et al., 2017).

In the present study, to efficiently balance pathways the IHTB strategy was established to fine-tune the (2S)-naringenin synthetic pathway based on a series of constitutive promoters with gradient strengths (S. Zhou, Ding et al., 2017). Three important benefits of the method presented are: (a) the strains constructed do not require the use of expensive inducers; (b) constitutive promoter libraries can avoid unexpected recombination events in expression cassettes compared with mutation-based promoter libraries (Montiel, Kang, Charlop-Powers, & Brady, 2015; S. Zhou, Ding et al., 2017); (c) comparing with RBS engineering, IHTB strategy regulate gene expression at transcriptional level and avoided overexpression of mRNA. In the IHTB strategy, the promoters that drive the expression of the (2S)-naringenin biosynthetic genes were sequenced and their transcriptional strengths were identified based on results presented in our previous study (S. Zhou, Ding et al., 2017). Finally, we speculated that the rate-limiting step of (2S)-naringenin synthesis involved CHS, instead of TAL as previously reported (Santos et al., 2011; J. Zhou et al., 2014; S. Zhou et al., 2016). This may be because a higher TAL activity level was used in the present study (Jendresen et al., 2015). The IHTB strategy allows more efficiently fine-tuning of multigene pathways by using a series of constitutive gradient promoters.

Unlike the mutation promoter libraries, which contain lots of nonfunctional variants, the promoter libraries used in this study have several obvious advantages, that is, small scale, high phenotype diversity and low homology. Therefore, the final pathway libraries, which carried different promoter-gene combinations, have a small size (~50,000), for which the multiwell plate-based screening method could already fulfill the requirement. With the development of high-throughput sequencing technology, many native promoters from diverse industrial microorganisms, including E. coli (S. Zhou, Ding et al., 2017), S. cerevisiae (Redden & Alper, 2015; Yuan et al., 2017), Bacillus subtilis (Song et al., 2016; Yang, Du, Chen, & Kang, 2017), Streptomyces (Li et al., 2015; Y. Luo, Zhang, Barton, & Zhao, 2015), and Hyperthermophilic archaeon (S. H. Lee et al., 2015), have been screened and characterized. These promoters provide the tools necessary to fine-tune multigene pathways by IHTB strategy. IHTB strategy not only decreased the overall workload but can also be used to construct single-plasmid systems using ePathBrick plasmids (P. Xu, Vansiri, Bhan, & Koffas, 2012). If necessary, the balanced pathway can be integrated into the genome to further reduce the metabolic burden (Englaender et al., 2017; Jones et al., 2015). Compared with traditional pathway balancing, computational-based pathway design (Long et al., 2015), and multimodular (Biggs et al., 2014) strategies, IHTB is a semirational pathway balancing method that can efficiently optimize the pathway within a wide range of expression levels without the need for detailed knowledge of pathway reaction parameters.

(2S)-Naringenin plays a crucial role in the synthesis of diverse flavonoid derivatives (Whitaker et al., 2017; J. Wu, Du, Zhou, & Chen, 2014). However, until recently, liquid chromatography has been the only method for assaying the (2S)-naringenin produced by engineered microbes, with a method that can be translated to high-throughput screening using riboswitches being only very recently described (Xiu et al., 2017). The complicated pretreatment procedure and long detection time required for HPLC (Santos et al., 2011; J. Wu, Zhou et al., 2014) limited the development of a high-throughput technology for enhancing (2S)-naringenin biosynthesis. Compared with the HPLC method, the greatest advantages of these methods are convenience, rapidity, and high-throughput capacities. Compared with riboswitch based high-throughput screening method, UVS- and FS-based assay methods are suitable for small scale library screening ($10^4$-$10^5$). The method did not require flow cytometry, which are rarely available to most of the labs related to metabolic engineering or synthetic biology. Although some spectrophotometric assay methods has been reported for determining the total flavonoid content (Chang, Yang, Wen, & Chern, 2002; Pekal & Pyrzynska, 2014), it is unclear that how different metal ions can react with (2S)-naringenin to generate specific spectral properties that could be applied for the quantification of (2S)-naringenin in culture broth. To establish a high-throughput screening procedure of (2S)-naringenin in culture broth, both metal ions and reaction conditions were systematically optimized.

We believe that many interesting chemicals will be produced using the IHTB strategy. Many methods for screening specialty chemicals, including L-3,4-dihydroxyphenylalanine (DeLoache et al., 2015), lycopene (X. Xu et al., 2016), N-acetyl glucosamine (S.-W. Lee & Oh, 2015), organic acids (Z. Luo et al., 2017; Zeng, Du, Chen, Li, & Zhou, 2015), fatty acids (Hoover et al., 2012), and antibacterial drugs (Huang, Sudibya, & Chen, 2011; Tan et al., 2013), have been established. Perhaps the greatest challenge in preforming IHTB is establishing a universally used high-throughput screening procedure. Inspired by previous biosensor studies (Liu et al., 2017; Qian & Cirino, 2016; Wang, Tang, & Yang, 2017; Xiong et al., 2017), combining the advantages of transcriptomic and biosensor technology will allow many inducible promoters to be screened and novel biosensors constructed that respond to different concentrations of target chemicals for the high-throughput screening of high-titer strains.

Multivariate modular metabolic engineering is a widely applied strategy for balancing multigene pathways (Biggs et al., 2014). One of the limitations of this method is the limited availability of promoters and plasmids. To thoroughly fine-tune metabolic flux, every gene needs to be expressed at a different level to identify its optimum expression for production purposes. The challenge, however, with such an approach is that a huge number of possible expression combinations will need to be screened. The reverse metabolic engineering strategy presented by IHTB in this study allowed the rapid screening of 4,800 single colonies which possibly carried different combinations of genes and promoters. After several rounds of screening, the titer of (2S)-naringenin reached 191.9 mg/L, which is 2.1-fold of that of a strain optimized using a modular strategy (J. Wu, Zhou et al., 2014). Thus, IHTB is a promising method with applications in the efficient metabolic balancing of multigene pathways.
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CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

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