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Design and Characterization of Biosensors for the Screening of Modular Assembled Naringenin Biosynthetic Library in *Saccharomyces cerevisiae*

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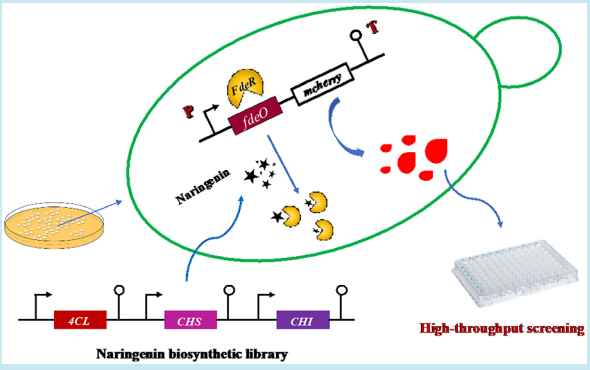
Supporting Information

ABSTRACT: A common challenge in the assembly and optimization of plant natural product biosynthetic pathways in recombinant hosts is the identification of gene orthologues that will result in best production titers. Here, we describe the modular assembly of a naringenin biosynthetic pathway in *Saccharomyces cerevisiae* that was facilitated by optimized naringenin-inducible prokaryotic transcription activators used as biosensors. The biosensors were designed and developed in *S. cerevisiae* by a multiparametric engineering strategy, which further was applied for the *in vivo*, high-throughput screening of the established yeast library. The workflow for assembling naringenin biosynthetic pathways involved Golden gate-directed combinatorial assembly of genes and promoters, resulting in a strain library ideally covering 972 combinations in *S. cerevisiae*. For improving the performance of our screening biosensor, a series of fundamental components was optimized, affecting the efficiency of the biosensor such as nuclear localization signal (NLS), the detector module and the effector module. One biosensor (*pTDH3*_NLS_FdeR-N_*tPGK1-pGPM1-fdeO*_mcherry_*tTDH1*-MV2) showed better performance, defined as better dynamic range and sensitivity than others established in this study as well as other previously reported naringenin biosensors. Using this biosensor, we were able to identify a recombinant *S. cerevisiae* strain as the most efficient candidate for the production of naringenin from the established naringenin biosynthetic library. This approach can be exploited for the optimization of other metabolites derived from the flavonoid biosynthetic pathways and more importantly employed in the characterization of putative flavonoid biosynthetic genes.



KEYWORDS: *Saccharomyces cerevisiae, biosensor, naringenin, modular assembly, flavonoids*

Plant secondary metabolites have been an important natural source of therapeutic molecules for the treatment of a variety of diseases as well as nutraceuticals. Among them, flavonoids are the largest group of plant polyphenolic compounds, with more than 9000 of these compounds identified so far.[1,2] On the basis of their chemical structure, flavonoids are classified into flavones, isoflavones, flavanones, flavonols, anthocyanins, and catechins.[2,3] The flavanone naringenin is one of the most essential intermediates for producing various flavonoids in the flavonoid biosynthetic pathway. In addition, naringenin has been shown to have valuable pharmacological activities for the treatment of diabetes, hypertension, obesity, and metabolic syndrome.[4−7]

Traditionally, flavonoids have been produced mainly by plant extraction, a method that can be limited by the availability of plant materials and requires large amount of extraction solvents. The development of plant cell cultures and microbial cell factories have emerged as promising and robust methods for producing high-value pharmaceutical and nutraceutical products.[8,9] Recently, heterogeneous production of flavonoids has been reported by refactoring biosynthetic pathways into engineered microorganisms such us *Escherichia*

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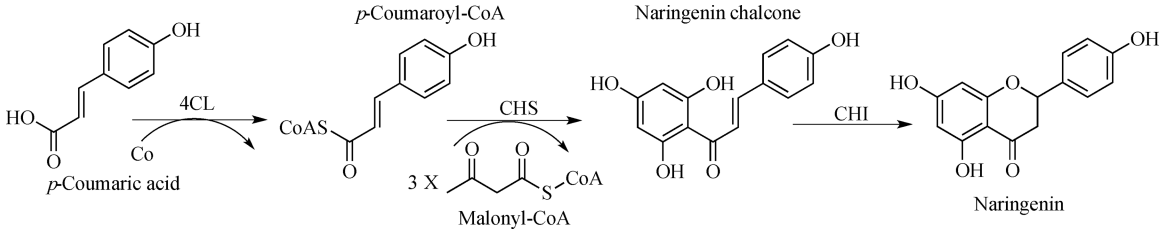

Figure 1. Schematic representation of the naringenin biosynthetic pathway from precursor *p*-coumaric acid. 4CL, *p*-coumaric acid CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase.

coli, *Corynebacterium glutamicum*, and *Saccharomyces cerevisiae*.[10−13] *S. cerevisiae* has been widely applied as a platform for the production of natural products due to several attractive characteristics.[14] First, the GRAS (generally recognized as safe) status and high tolerance against harsh industrial conditions are beneficial for producing food additives in large-scale bioreactors.[15] Second, it has been already studied in-depth as a model eukaryotic system, and as such it is a genetically tractable microorganism.[16] Third, its eukaryotic nature may be conducive to functional expression of plant-derived genes.[17]

Production of target chemicals of interest in recombinant cells usually requires complicated multistep bioreactions involving several enzymes in the introduced heterologous pathways. Hence, it is always critical to obtain enzymes with suitable enzymatic properties.[18] Specifically, in the case of the biosynthesis of plant secondary metabolites, such as flavonoids and stilbenes, it has repeatedly been shown that selection of the gene orthologues to be used in the metabolic pathway assembly plays a tremendous role in the final titers.[19,20] In addition, selection of promoters is an important parameter in ensuring proper expression of multiple genes.[21] As a result, combinations of heterologous gene orthologs and promoters with different strengths need to be explored for maximizing the metabolic fluxes through a recombinant pathway of interest. Such combinations are also important in order to achieve metabolic pathway balancing that usually requires additional experimental approaches that include post-translational balancing, DNA copy number modulation, dynamic balancing, promoter engineering, and RBS engineering.[22] As a result, a large phenotypic space needs to be explored in order to identify high producers. Development of high-throughput screening methods remains a serious challenge especially for metabolites whose production is not associated with color intensity changes. However, living organisms have evolved various mechanisms for monitoring the accumulation of metabolites in living environment.[23] Recently, ligand-inducible transcriptionally regulated biosensors have been developed as biosensors that can facilitate high-throughput screening of microbial production libraries.[22,23] To meet current demand, a few biosensors have been generated by modifying and introducing transcriptional regulators that have been identified in plant symbiotic bacteria and other organisms into industrial model strains.[26−28] In the case of flavonoids, such transcription-factor based biosensors have been developed for the molecules kaempferol, resveratrol, and naringenin and have found applications in engineering prokaryotic production platforms.[24,25] In addition, a naringenin-responsive biosensor was successfully constructed through directly transplanting prokaryotic transcription activator in the budding yeast *S. cerevisiae*.[29] For the development of a transcription biosensor, two basic modules are necessary, namely, a detector module and an effector module. The detector module is mainly composed of a promoter and an allosteric DNA-binding transcription factor coding sequence. The effector consists of the coding sequence of an output signal, such as fluorescent protein, and a modified promoter with the associated transcription factor binding sites. In high-throughput screening experiments, these two modules are associated with the function of biosensors reflected by a biosensor response curve that indicates the sensitivity or affinity of a biosensor to a ligand of interest.[30] Therefore, for the development of an efficient biosensor system, it is necessary to design and characterize both modules.

In this study, a *S. cerevisiae* naringenin-producing strain library was modularly established that covered around 1000 different combinations consisting of orthologs of flavanone biosynthetic genes and various associated biological regulating parts at the DNA and RNA levels. A naringenin-responsive prokaryotic transcription activator was designed and developed as a functional biosensor in *S. cerevisiae* using a multiparametric engineering strategy. After a series of optimizations of promoters, nuclear localization signals (NLS) and copy numbers, the biosensor S3-001-GPM1-MV2 (*pTDH3*_NLS_FdeR-N_*tPGK1-pGPM1-fdeO*_mcherry_*tTDH1*-MV2) showed a better dynamic range and sensitivity than other previously reported naringenin biosensors. This sensor was further successfully applied in high-throughput screening of the naringenin-producing strain library, and strain H2−2 was identified with a naringenin titer of 52.0 mg/L. Our study contributes to the construction of desired strain libraries by random modular assembly and screening using transcription factor-based biosensors in *S. cerevisiae*.

RESULTS AND DISCUSSION

Selection of Naringenin Biosynthesis Genes. As shown in Figure 1, naringenin biosynthesis occurs *via* a malonyl-CoA dependent pathway from precursor *p*-coumaric acid (*p*-CoA) involving three key enzymes, namely, 4CL (4-coumaric acid-CoA ligase), CHS (chalcone synthase), and CHI (chalcone isomerase). Despite extensive work on the recombinant biosynthesis of flavonoids, it is still not clear which one of the three enzymes is the rate-limiting step in this biosynthetic pathway. In order to identify gene orthologs that encode for enzymes with more favorable biochemical properties, naringenin biosynthetic genes (two 4CL, three CHS, and two CHI) from different plant sources were selected and introduced in *S. cerevisiae* for heterologous overexpression. The selection was made based on previous reports of the biochemical properties of these enzymes, such as the ratio of k_{cat}/K_M. The DNA sequences and other information regarding

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the naringenin biosynthetic enzymes used in this study are shown in supplementary data and Table 1.

Table 1. Naringenin Biosynthetic Enzymes Used in This Study

name	Uniprot ID	gene source	enzyme classification
At4CL2	Q9S725	*Arabidopsis thaliana*	4-Coumaric acid-CoA ligase
Pto4CL4	H9AZ23	*Populus tomentosa*	
PpCHS	Q2VAZ3	*Physcomitrella patens*	Chalcone synthase
HaCHS	Q9FUB7	*Hypericum androsaemum*	
MsCHS2	P30074	*Medicago sativa*	
PhCHI	P11650	*Petunia hybrida*	Chalcone isomerase
GmCHI2	Q53B74	*Glycine max*	

Modular Assembly of Naringenin Biosynthetic Libraries. The combinatorial assembly of the naringenin biosynthetic library is based on Golden Gate cloning and involved three steps. First, all selected candidate naringenin biosynthetic genes were synthesized after removing unique restriction sites (*BsaI*, *BsmBI*, and *NotI*) and appending specific DNA fragments that were designed based on the instructions of MoClo yeast toolkit. The resulting DNA fragments were assembled into the universal entry vector, pYTK001 using *BsmBI*, resulting in S1_At4CL2, S1_Pto4CL4, S1_PpCHS, S1_HaCHS, S1_MsCHS2, S1_PhCHI, and S1_GmCHI2. Lastly, the above plasmids were assembled into vector pYTK095 together with associated plasmids shown in Table S1 by *BsaI* assembly, resulting in plasmids S2_028 to S2_048 (Figure 2A).

An appropriate promoter is necessary in order to balance the expression of the heterologous or endogenous genes that lead to the synthesis of desired products in metabolic engineering. It has been shown in the recent past that when it comes to secondary metabolites such balancing is essential, and oftentimes leads to counterintuitive results in which weaker promoters result in better production titers.[31,32] In the case of yeast, there are three different types of yeast promoters, namely, constitutive promoters, inducible promoters, and mating-type-specific promoters. Recently, an increasing amount of effort has been placed on the characterization of yeast promoters.[33−35] In this study, every possible pair of the selected naringenin biosynthetic genes with each of the three promoters with different expression strengths (strong, intermediate, and weak) was constructed. In addition, it was previously shown that another important parameter that affects gene transcription levels is the expression system (copy number of genes), typically including genome integration (single copy) plasmids, low-copy CEN/ARS4 plasmids (1−4 copies/cell), and high-copy 2 μm plasmids (20−30 copies/cell).[16] Several studies have already demonstrated that higher-copy number plasmids are beneficial for increasing heterologous gene expression levels.[36,37] However, prior research has shown higher gene expression using low-copy CEN/ARS4 plasmids than high-copy 2 μm plasmids.[16] Therefore, the effect of gene copy number on heterologous pathway expression is unpredictable and should be evaluated case by case. In our study, as shown in Figure 2B, versions of step two cassettes

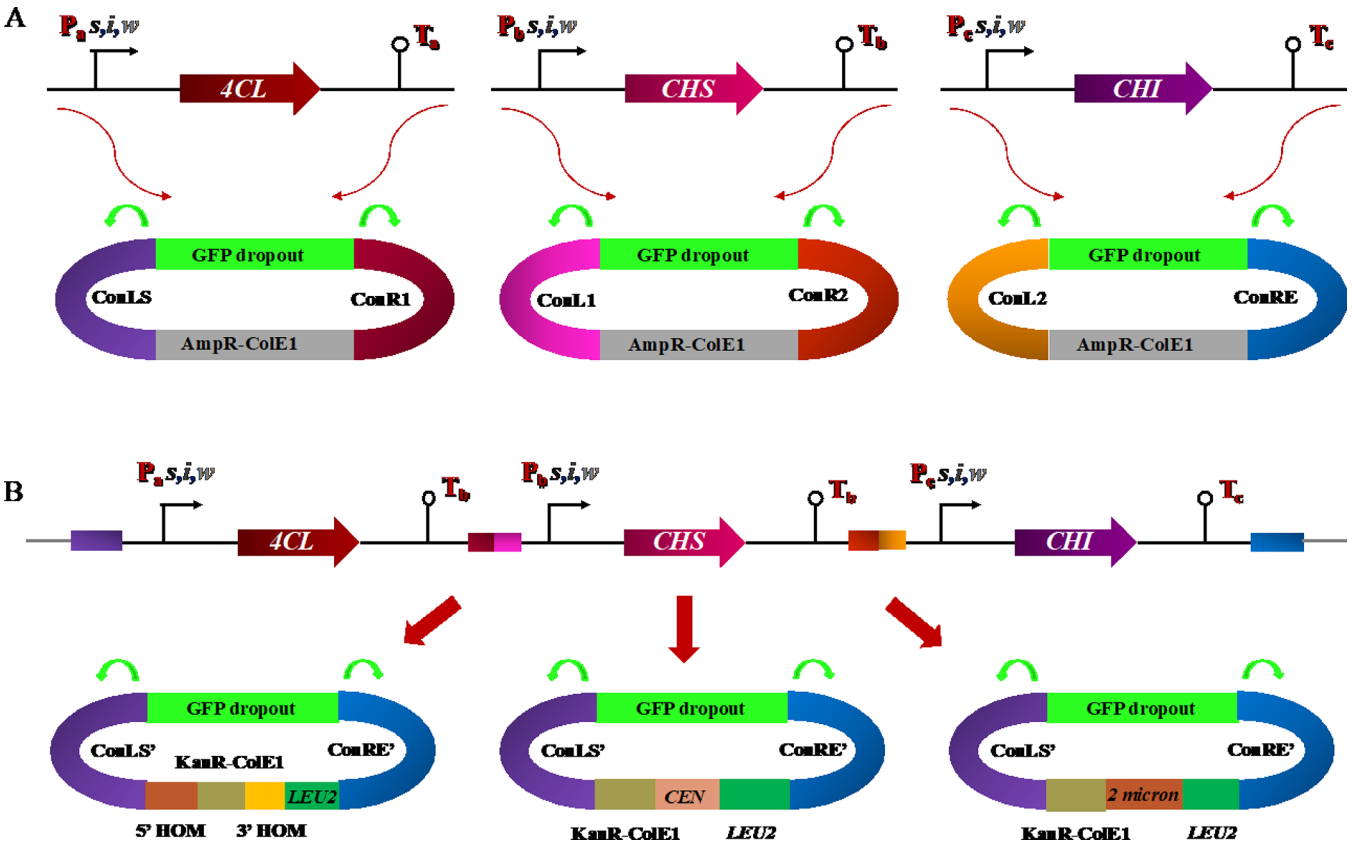

Figure 2. Naringenin biosynthetic libraries generated by Golden gated-directed combinatorial assembly. P_s, P_i, P_w represent promoters with strong, intermediate, and weak strengths, respectively.

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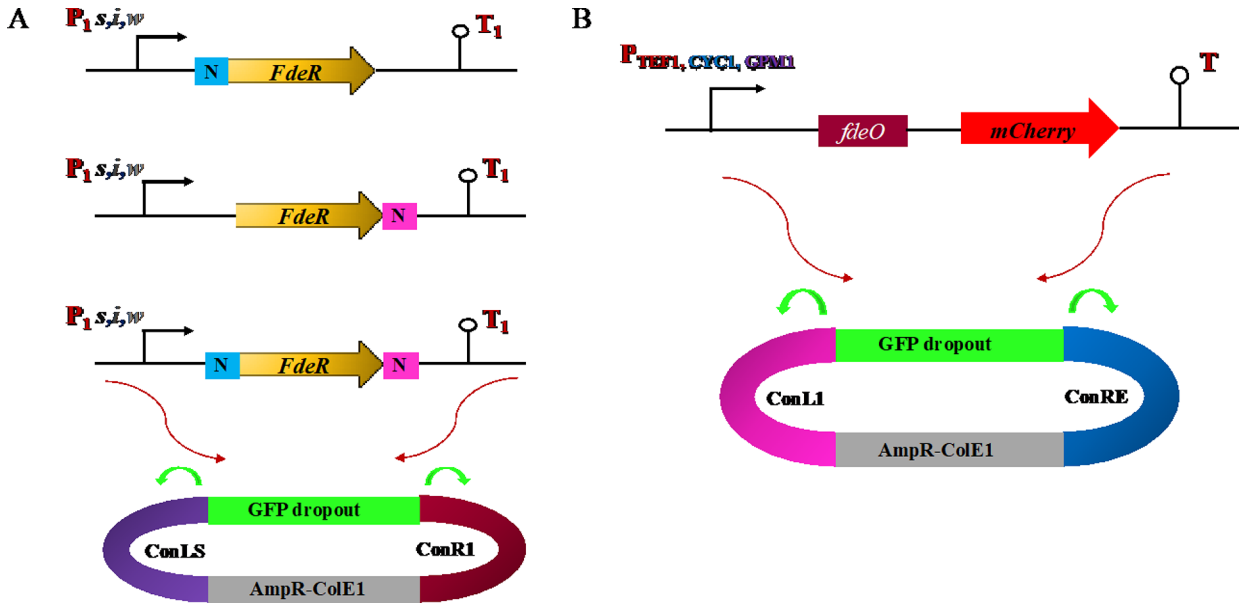

Figure 3. Schematic representation of the design of a naringenin-responsive transcription biosensor with associated detector (A) and effector module (B).

were constructed in three different copy number vectors. Finally, a random naringenin pathway biosynthetic library covering 972 combinations was successfully constructed in *S. cerevisiae* by Golden gate cloning-directed combinatorial assembly.

Selection of Platform Strains. In order to identify the best host for naringenin production, three different *Saccharomyces cerevisiae* reference strains (SCE328, CEN.PK2−1C, and INVSc1) were evaluated. The high-copy number plasmid pYTK098-*pCCW12*_At4CL2_*tADH1-pPGK1*_*HaCHS*_*tENO2-pHHF2*_*PhCHI*_*tSSA1* (pYTK098-S2_028_S2_037-S2_043) was constructed and transformed into these three strains, resulting in three naringenin producing strains NGN-01, NGN-02, and NGN-03. According to the HPLC results, all of these strains had the capability to produce naringenin (Figure S1). However, at least one additional byproduct was identified in the fermentation broths of NGN-01 and NGN-03 that was absent from the fermentation broth of NGN-02. For this reason, we chose strain CEN.PK2−1C as the host for naringenin production in our subsequent experiments.

Design of the Naringenin Sensor in *S. cerevisiae*. So far, two native naringenin-responsive regulatory systems have been identified and applied in heterologous hosts, namely LysR-type PfdeAR-FdeR from *Herbaspirillum seropedicae*, and TeTR-type PttgABC-TtgR from *Pseudomonas putida* DOT-T1E.[24,25] Several fundamental components should be taken into account to establish an ideal biosensor in *S. cerevisiae*, such as nuclear localization signal (NLS), transcription factor (TF), and an effective modified promoter with specific binding site. Here, we established and characterized a series of sensors to develop and optimize a naringenin-responsive one with desired properties.

As an important component of a biosensor designed for yeast, NLS was used to direct the sensor to the cell nucleus. Different nuclear localization signals can change the rate of binding of operator and transcription factor. In *S. cerevisiae*, two types of NLS have commonly been used: c-Myc and SV40. Considering the potential effect on the binding rate, we chose the strong SV40 and compared three combinations to ensure transport into the nucleus by varying the location of the NLS; specifically we tested its effect when SV40 was located at the sensor's N-terminus, C-terminus, and both (Figure 3A). The transcription factors FdeR and TtgR were transformed into the budding yeast under the control of three different yeast constitutive promoters with varying strengths: *pTDH3* (strong), *pRPL18B* (intermediate), and *pREV1* (weak), resulting in plasmids S2_001 to S2_018 (Figure 3A and Table S1). On the basis of a previous report that developed a naringenin-inducible sensor in yeast by introducing a 73 bp *fdeO* operator (*fdeO_K*) in *pCYC1* promoter, we followed a similar approach and introduced the same binding site in *pCYC1* and *pGPM1* promoters. Furthermore, in our effort to improve the properties of the engineered promoters, a shorter FdeR-specific binding site, the 49 bp *fdeO* operator, was introduced in *pGPM1* as well as the yeast constitutive promoter *pTEF1* (Figure 3B and Figure 4). In addition, the 51 bp *ttgA* operator was inserted after promoters *pCYC1*, *pTEF1*, and *pGPM1*, respectively. The above-engineered promoters only contained a single relative operator, except

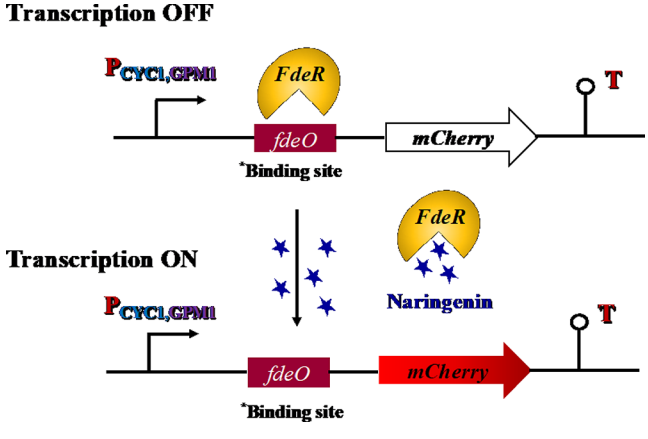

Figure 4. Schematic views of FdeR-based regulation system in yeast.

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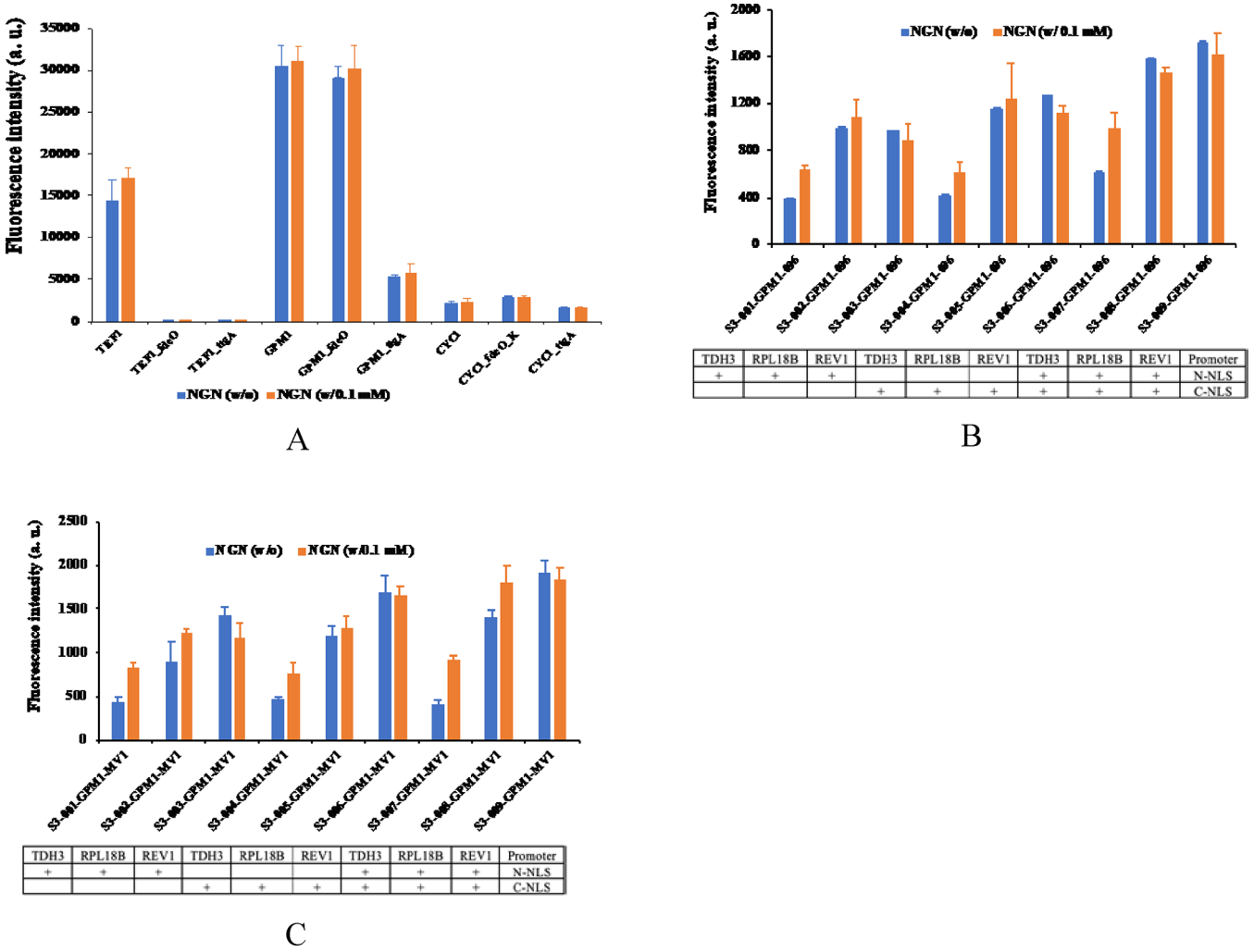

Figure 5. Effects of inserted operator on the behavior of selected promoters (A). Fluorescence intensity comparison of integration type biosensors (B) and low-copy number plasmid type biosensors (C) with various NLS in the presence or absence of 0.10 mM naringenin. The 096 and MV1 represent genome integration type and low-copy number type. N-NLS and C-NLS indicate the location of NLS at N-terminus and C-terminus of FdeR-N, respectively. "+" means which type NLS was in biosensor.

for *pTEF1* that contained three *fdeO* operators inserted at different locations (supplementary ordered sequences).

Characterization of the Naringenin Sensor in *S. cerevisiae*. To investigate the effects of inserted operators on the behavior of the promoter, strains harboring plasmids expressing the fluorescent protein mCherry with original or engineered promoters were constructed and compared. As shown is Figure 5A, the FdeR-regulated mCherry expression cassette with modified *TEF1* promoter was completely inactive. The addition of naringenin at a final concentration of 0.10 mM did not alter the fluorescence intensity on all promoters tested. The insertion of *ttgR* binding sites resulted in a decrease of fluorescence intensity in the case of the *GPM1* and *CYC1* promoters, while there was no significant difference between the above original promoters and modified promoters *GPM1_fdeO* and *CYC1_fdeO_K*. This indicated that the engineered *GPM1* and *CYC1* promoters exhibited a potential to be further used for the development of ligand-inducible biosensors. In addition, it was found that the fluorescence intensity of the strain expressing mCherry under the *GPM1* promoter was much higher than the CYC1 one, implying that the engineered *GPM1* promoter was stronger. Finally, a detector module was cloned in an integration plasmid as well as a low-copy number plasmid.

In Figure 4, the schematic views of the FdeR sensor system in yeast is presented. This system is based on the naringenin-sensitive transcription factor (FdeR) and the binding sites (*fdeO*) integrated into a specific promoter (*GPM1* or *CYC1* promoter) to control fluorescence protein (mCherry) expression as output signal. The more naringenin is produced inside the cell, the more FdeR and subsequently mCherry is expressed. A series of constructs with varying NLS and constitutive promoters of different strengths were tested; however, no activity was observed in strains harboring TtgR as detector module (data not shown). Therefore, we decided to only focus on FdeR as detector module in subsequent experiments.

We constructed and compared the fluorescence intensity of 18 sensor strains in minimal medium with or without 0.10 mM of naringenin, in order to determine the effects of NLS location, promoter strength, and copy number either on gene transcription level or protein expression level. As shown in Figure 5B,C, both integration and low-copy number sensor strains showed similar results: in both cases the strong *pTDH3* promoter was optimal, based on the dynamic range of the

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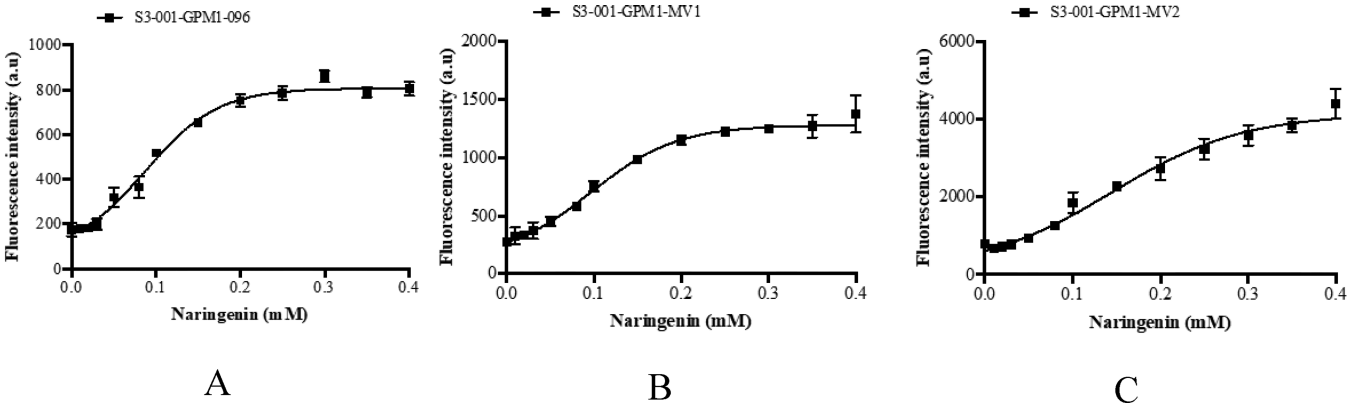

Figure 6. Dynamic response of sensors designed with *pTDH3*_NLS_FdeR-N_*tPGK1* (001) and *pGPM1-fdeO*_mcherry_*tTDH1* (GPM1) cassette using three different types of vectors: Integration, 096 (A), Low-copy number, MV1 (B), and High-copy number, MV2 (C).

sensors' response to naringenin. But expression of FdeR using either intermediate strength *pRPL18B* or weak *pREV1* promoters did not result in significant change or increase of mCherry fluorescence intensity, which was in accord with the previous report.[29] Meanwhile, the strong promoter sensor strains had less fluorescence intensity than the ones using weaker promoters. The low concentration of FdeR in weaker promoter strains resulted in high background "leaky" expression.[38] We speculated that stronger promoter was more beneficial for expressing transcription factor FdeR, resulting in stronger transcription activation of the effector module. However, there was not enough evidence to estimate which NLS type was the optimal according to the results of Figure 5B,C.

We further focused our efforts on the sensitivity assessment and operational range of the naringenin sensors using the *pTDH3*-FdeR cassette by determining the dose-dependent response curves before applying biosensors for detection of naringenin *in vivo*. For S3-001-GPM1-096, (*pTDH3*_NLS_FdeR-N_*tPGK1-pGPM1-fdeO*_mcherry_*tTDH1*-096) S3-001-GPM1-MV1 and S3-001-GPM1-MV2, all of these three N-terminus NLS type sensor systems showed a gradual response corresponding to naringenin concentration following 12 h of cultivation (Figure 6). The ratio of fluorescence intensity of chromosomally integrated construct strain S3-001-GPM1-096 increased from 1 to 4.7, and plateaued when naringenin concentration reached 0.20 mM; a similar result was observed with low-copy number strain S3-001-GPM1-MV1. The high-copy number strain S3-001-GPM1-MV2 showed a higher dose-dependent response to naringenin concentration, and reached saturation at concentrations above 0.40 mM. For strains carrying the NLS signal either at the N-terminus or at both ends of the sensor, high-copy number expression resulted in more sensitive response and broader dynamic range (Figure S1 and S2). We also found that when the NLS was inserted at the C-terminus, the resulting sensor exhibited the lowest dose-dependent response. For instance, the saturation concentration of naringenin occurred between 0.15 and 0.20 mM in S3-004-GPM1-MV2 (*pTDH3*_FdeR-N_2×NLS_*tPGK1-pGPM1-fdeO*_mcherry_*tTDH1*-MV2, Figure S1). So far, there was only one report on the naringenin responsive biosensor for yeast that only showed about 1.7 fold increase in the presence of 0.20 mM naringenin and the dynamic range was determined from 0 mM to 0.20 mM naringenin. The biosensor S3-001-GPM1-MV2 in our study represented almost 3.0 fold increase under the same concentration and its dynamic range was detected from 0 mM to 0.40 mM naringenin. Therefore, S3-001-GPM1-MV2 could serve as a better sensor due to the relatively broad dynamic range and sensitivity.[25]

To further investigate the effects of different operators on the response of the biosensor, we replaced *fdeO* with operator *fdeO_K* and constructed corresponding strains with different copy numbers, S3-001-fdeO_K-096, S3-001-fdeO_K-MV1, and S3-001-fdeO_K-MV2 (*pTDH3*_NLS_FdeR-N_*tPGK1-pGPM1-fdeO_K*_mcherry_*tTDH1*-MV2). Similarly, we determined the dynamic range of these three sensors using different concentrations of naringenin. We noticed that only 001-fdeO_K-096 strain showed a dose-dependent response to a series of naringenin concentrations and the saturation concentration was 0.15 mM of naringenin (Figure S3A). Compared to the strain grown without naringenin, the fluorescence intensity was enhanced about 4 fold in 001-fdeO_K-096 strain supplemented with 0.15 mM of naringenin. For strains S3-001-fdeO_K-MV1 and S3-001-fdeO_K-MV2, we did not observe any dose-dependent response between fluorescence intensity and applied concentrations of naringenin (Figure S3B,C). In our study, the above results indicated that the shorter *fdeO* operator that was first used in biosensor was more suitable than *fdeO_K* and demonstrated the critical role of the operator in the binding efficiency of the FdeR biosensor. As such, the modification of the operator sequence by shortening its length and altering its sequence can provide an alternative strategy for tuning the performance of a biosensor to current efforts that focus on computational protein design.[39]

The detector module and effector module in all biosensors we constructed were assembled in one vector, in order for both modules to have the same gene copy number. Furthermore, we used the high-copy number plasmid pYTKV004 to clone the NLS-FdeR construct; this construct was used for all subsequent experiments. At the same time, we assembled promoter GPM1 modified with *fdeO* or *fdeO_K* operators and mCherry gene into three types of vectors with different copy-numbers. Considering that gene dosage is an important factor that determines the performance of biosensors, we constructed the following six strains by transforming the corresponding plasmids in yeast: S3-GPM01r-fdeO_K-096, S3-GPM01r_fdeO_K-MV1, S3-GPM01r_fdeO_K-MV2, S3-GPM01r-fdeO-096, S3-GPM01r-fdeO-MV1, and S3-GPM01r-fdeO-MV2. As shown in Figure S3 and S4, biosensors with *fdeO* and *fdeO_K* exhibited similar dose-

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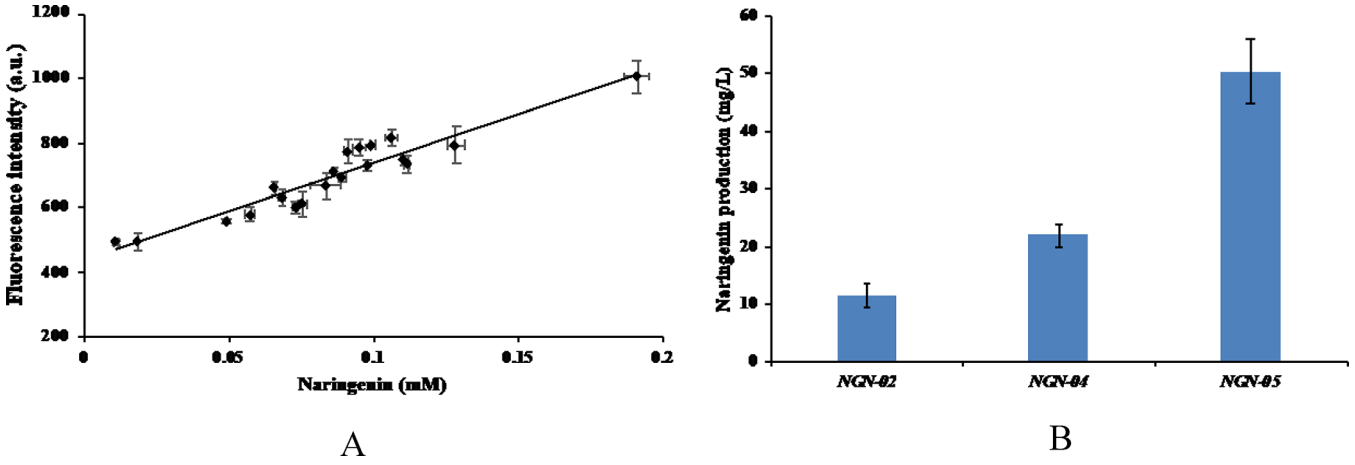

Figure 7. Correlation between concentration of naringenin produced and relative fluorescence intensity (A). Naringenin productions from strains (NGN-02, NGN-04, and NGN-05) expressing the naringenin biosynthetic pathway containing different promoters (*pCCW12*, *pALD6*, and *pRAD27*) for 4CL (B).

dependent responses to various concentrations of naringenin under the control of integration or high-copy number vector. The fluorescence intensity of S3-GPM01r-fdeO-MV1 had a sharp increase in a narrow concentration range of naringenin (0.075 to 0.15 mM). Compared with the biosensor S3-001-GPM1-MV2, there were no observed advantages with respect to the biosensor sensitivity and operation range when the detector module and effector module were established separately in different plasmids. The above results indicated that the effects of gene copy number for both the transcription factor and the operator should be investigated and compared in order to optimize the performance of the biosensor in yeast.

***In Vivo* Screening Application of Naringenin Biosensor in *S. cerevisiae*.** For assembling the naringenin biosynthetic libraries, the ideal situation would be that three vectors (pYTK097, pYTK098, and pYTKV015) should be randomly combined with naringenin biosynthetic genes, resulting in plasmids covering all potential 972 combinations. To validate the applicability of established naringenin-inducible biosensors for high throughput screening in *S. cerevisiae*, randomly constructed plasmids carrying genes for the naringenin biosynthetic pathway were transformed into the biosensor S3-001-GPM1-MV2. In the beginning, 20 yeast strains named T1 to T20 were randomly selected and cultivated for 24 h to detect the fluorescence signals, and the corresponding naringenin concentration in the medium was further analyzed by HPLC following 48 h of cultivation.

As shown in Figure 7, the naringenin-inducible biosensor had a good correlation (r = 0.95) between mCherry fluorescence intensity output and naringenin titers, spanning a range of 0.011 to 0.19 mM (3.05 to 52.0 mg/L). The above preliminary validation results not only indicated the potential of using the naringenin biosensor for *in vivo* screening of naringenin-producing *S. cerevisiae* recombinant strains, but also proved that hierarchical levels of biological parts (*i.e.*, plasmid copy number engineering, promoter engineering and protein engineering) should be well-characterized and optimized for metabolic engineering applications. The distribution of naringenin production indicated that the established library was as broad as we expected. Furthermore, this naringenin biosensor was utilized for screening the entire naringenin biosynthetic library. The biases in the construction were not noticed among the 20 strains (Figure 7A). Finally, the entire established library including about 2500 strains was screened, and two strains (H2−2 and H4−6) from this entire library screening that showed no significant differences on mCherry fluorescence intensity output with the highest naringenin titer (T5, 52.0 mg/L) in the above preliminary experiments (Figure 7B). Hence, all of these three strains were chosen as the candidates for extracting plasmids and gene sequencing. The sequencing results showed that all three strains carried the same plasmid, namely, pYTK098-S2_030-S2_037-S2_043 (high copy number plasmid with *pRAD27*-At4Cl2, *pPGK1*-HaCHS, *pHHF2*-PhCHI).

In the construction of naringenin pathway, three promoters (*pCCW12*, *pALD6*, and *pRAD27*) have been used to modulate the expression of *p*-coumarate acid CoA ligases. The relative strength of promoter *pRAD27* is the lowest in the above-mentioned promoters. To confirm whether *pRAD27* was the most appropriate promoter in this case, we further constructed plasmid pYTK098-S2_029-S2_037-S2_043, obtained the associated strain NGN-04 and compared naringenin production of strains (NGN-02, NGN-04, and NGN-05) expressing 4CL with the above three promoters. As shown in Figure 7B, naringenin production decreased with increasing promoter strength, and the naringenin production of NGN-05 with promoter pRAD27 was about 5-fold of NGN-02 containing promoter *pCCW12*. These results indicated that appropriate strength promoters and befitting enzymes should be comprehensively considered when it comes to the application of pathway engineering in *S. cerevisiae*.

In this study, a random naringenin biosynthetic library was modularly established in *S. cerevisiae*. The overall design of this library was quite easy to follow and contributes to the construction of other desired strain libraries involving in different orthologs of biosynthetic genes and various biological regulating parts at the DNA and RNA levels in *S. cerevisiae*. A naringenin-responsive prokaryotic transcription activator was designed and developed as a functional biosensor with a shortened binding site in *S. cerevisiae* using multiparametric engineering strategy. We have comprehensively considered and confirmed the critical roles of different fundamental biological regulating components in the construction of naringenin-responsive biosensors for yeast, such as promoters, nuclear localization signals (NLS) and copy numbers, *etc*. Finally, the optimized biosensor, S3-001-GPM1-MV2, showed better

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potential performance on dynamic range and sensitivity than others established in this study as well as other previously reported naringenin biosensors. We described that the well-characterized naringenin-inducible biosensor system based on the transcription regulator FdeR can be used to screen *in vitro* or *in vivo* for the best-performing biocatalysts and developed efficient yeast cell factories with optimized pathways for natural product synthesis. Since naringenin is one of the most important intermediates in the synthesis of flavonoids, we also expect that the designed biosensor can be exploited for high-throughput screening of yeast strain libraries involving genetic designs for the production of other flavonoid molecules that are derived from naringenin.

In conclusion, our results demonstrate that random modular assembly of plant secondary metabolic pathways in yeast can help identify the genetic elements that result in higher production titers. For screening the resulting libraries, appropriate sensors with high sensitivity are necessary. We demonstrated that constructing and optimizing a well-characterized and optimized naringenin-responsive sensor involved testing and characterizing various components. The ability to apply heterologous transcriptional regulators in *S. cerevisiae* for monitoring specific compounds, and in particular of the naringenin-inducible prokaryote transcription activator as a biosensor, will facilitate the development of efficient recombinant cell factories for the production of target compounds.

MATERIALS AND METHODS

Strains and Plasmids. All strains, vectors and plasmids used in this study are listed in Supplementary Table S1. *E. coli* DH5α was used for recombinant DNA manipulation. *S. cerevisiae* strains SCE328, INVSc1, and CEN.PK2−1C were used as the hosts for naringenin production. Strain CEN.PK2−1C also was used for gene cloning and biosensor validation. The plasmids of MoClo yeast toolkit for cloning and DNA assembly were acquired from Addgene.[35]

Chemicals and Cultivation Conditions. Authentic standards of *p*-coumaric acid and naringenin were purchased from Sigma-Aldrich (St. Louis, MO, USA). *E. coli* DH5α was grown at 37 °C in Luria−Bertani broth (LB) medium containing the appropriate antibiotics (25 mg/L chloramphenicol, 80 mg/L ampicillin, and 100 mg/L kanamycin) when required. Synthetic complete (SC) dropout media were used for the selection of recombinant yeast strains, consisting of 6.7 g/L of Yeast nitrogen base (YNB) without amino acid and ammonium sulfate (Sigma-Aldrich), 2% glucose, 0.79 g/L of appropriate complete supplement mixture (CSM) (Sunrise, San Diego, CA, USA), and 0.5% ammonium sulfate. For sensor evaluation, yeast strains were cultured in minimal medium (MM) with 20 g/L of dextrose, 7.5 g/L of $(NH_4)_2SO_4$, 14.4 g/L of KH_2PO_4, 0.5 g/L of $MgSO_4{\cdot}7H_2O$, 2 mL/L trace metals solution, and 1 mL/L vitamins solution. Appropriate amino acids or supplements to the auxotrophic requirements of yeast strains were supplied into mineral medium at 150 mg/L for uracil, 500 mg/L for leucine, 125 mg/L for histidine, and 75 mg/L for tryptophan.[17]

DNA Manipulation. All oligonucleotide primers used to amplify DNA fragments are shown in Tables S2 and S3, respectively. Synthetic genes were commercially synthesized by IDT (Skokie, IN, USA) and GeneScript (Piscataway, NJ, USA) and their sequences are provided in Supporting Information. ACCUZYME DNA Polymerase (Bioline, London, UK) was used for PCR reactions. The *mCherry* gene was amplified from the pTH761-CEN-mCherry_v4 plasmid purchased from Addgene. Yeast transformation was conducted by the standard lithium acetate method.[40] Yeast plasmids were extracted by Zymoprep Yeast Plasmid Miniprep II kit (Zymo Research, Irvine, CA, USA). DNA modular construction and multipart assembly were performed by a highly characterized yeast toolkit.[35] Golden Gate cloning reaction mixture used for assembling plasmids in this study was prepared as follows: appropriate concentration of DNA insert or plasmid (the ratio of DNA insert to backbone was 40:20 fmol), 0.25 μL of T7 DNA ligase (NEB, Ipswich, MA, USA), 0.5 μL of 10 mM ATP, 0.25 μL of 100 mM DTT, 0.4 μL of BsmBI or BsaI (10 000 U/mL from NEB), 0.5 μL of 10× 3.1 buffer (for BsmBI) or 10× CutSmart Buffer (for BsaI), and water to bring the final volume to 5 μL. After mixing well by pipetting, the mixtures were incubated in a PCR thermocycler as follows: 30 cycles of digestion and ligation at 42 °C (37 °C for *BsaI*) for 5 min and 16 °C for 5 min respectively were followed by a final digestion at 55 °C for 30 min, and then a heat inactivation for 10 min at 80 °C. After incubation of Golden Gate, Plasmid-Safe was used as the final purification step as follows: 0.75 μL of 10 mM ATP, 0.75 μL of Plasmid-Safe ATP-Dependent DNase (10 U/μL, Epicenter, Madison, WI, USA), 0.75 μL of Plasmid-Safe 10× reaction buffer, and 0.75 μL of sterile water. The DNA fragments of NLS and 2×NLS were annealed using designed oligos according to the protocol of T4 Polynucleotide Kinase (NEB).

Characterization of Naringenin Sensor in *S. cerevisiae*. The mCherry fluorescence and OD_{650} measurements were performed on a SynergyMx multimode microplate reader (BioTek, Winooski, VT, USA) with the excitation at 587 nm and the emission at 610 nm.[41] Fluorescence intensity (a.u.) was normalized to cell density. Unless otherwise noted, precultures derived from single yeast colonies were incubated in 1 mL of minimal medium supplemented with 20 g/L glucose at 30 °C and 250 rpm for 18 h in 48 deep-well plates (VWR, Radnor, PA, USA). For monitoring the influence of naringenin on mCherry fluorescence, yeast cells were grown at an initial OD_{650} of 0.1 in 2 mL of fresh medium with different concentrations of naringenin (0, 0.01, 0.02, 0.03, 0.05, 0.08, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40 mM) in 48 deep-well plates for 12 h.

Library Construction and High-Throughput Screening. The library of naringenin producing strains was prepared as follows (Figure 2): all the naringenin biosynthetic candidate genes (Table 1) were cloned into the entry vector pYTK001 following the yeast Golden gate assembly protocol described above. These candidate genes were further inserted into the second vector pYTK095 with various promoters and terminators to construct plasmids S2_028 to S2_048 shown in Table S1. Next, Plasmids carrying 4CL orthologs (S2_028 to S2_033), CHS orthologs (S2_034 to S2_039) and CHI orthologs (S2_043 to S2_048) were mixed and inserted into the following three yeast expression vectors: pYTKV015 (Integration vector), pYTK097 (Low copy number vector) and pYTK098 (High copy number vector), respectively. Next, the golden gate reaction mixtures were directly transformed into *E. coli* DH5α using Plasmid-Safe kit and were grown on LB plates supplemented with 100 mg/L of kanamycin. Plasmids carrying 4CL orthologs (S2_028 to S2_033), CHS orthologs (S2_040 to S2_043), and CHI orthologs (S2_043 to S2_048) were prepared the same way as previously

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described. Plasmids of *E. coli* transformants grown on LB plates were isolated using E.Z.N.A. Plasmid Mini Kit I (Omega, Norcross, GA, USA) and were transformed into the sensor strain. The resulting transformation mixtures were plated on SC plates lacking Ura and Leu.

Host cells transformed with various plasmids were cultivated for 20 h in 1 mL of SC dropout media supplemented with 20 g/L glucose at 30 °C and 250 rpm in 48 deep-well plates. Then yeast cells were inoculated in 2 mL of corresponding fresh mineral medium with 1.0 mM of *p*-coumaric acid at an initial OD_{650} of 0.1 in 48 deep-well plates for 48 h.

Metabolite Quantification Using HPLC. For the naringenin production yeast strains, 200 μL fermentation broth was extracted with an equal volume of methanol, vortexed for 10 s, and centrifuged at 20 000*g* for 15 min. Twenty-five μL of supernatant was further analyzed by Agilent 1200 series (Agilent, Santa Clara, CA, USA) equipped with a ZORBAX SB-18 column (4.6 × 150 mm, 5 μm particle size, Agilent) connected to a diode array detector. A flow rate of 1.0 mL/min was performed with acetonitrile containing 0.1% formic acid (phase A) and water containing 0.1% formic acid (phase B) using the following gradient elution program: 10−40% A (0−10 min), 40−60% A (10−15 min). A calibration curve of authentic standard was used for quantification. All experiments were performed in triplicates.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.9b00212.

Tables S1−S4; Figures S1−S4 (PDF)

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R.W., B.F.C., Z.W., and M.A.G.K. conceived this study. R.W., Z.Y., and J.C.H. conducted the experiments. R.W. and S.Z. performed data analysis. R.W., B.F.C., Z.W., and M.A.G.K. wrote this manuscript. G.Y.J., Z.W., and M.A.G.K. supervised the projects. All authors edited the manuscript.

Notes

The authors declare no competing financial interest.

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