Directed Evolution of Chalcone Synthase to Enhance Production of Naringenin and Sakuranetin with Biosensor in *Vibrio natriegens*

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Abstract: The synthesis yield of sakuranetin, a compound with extensive medical applications and potential, is constrained by the condensation reaction mediated by chalcone synthase. In recent years, directed evolution has emerged as a promising approach for enhancing protein performance; however, traditional directed evolution techniques encounter challenges in high-throughput screening. By integrating rational design and protein structure analysis, we have modified chalcone synthase and the naringenin sensors TtgR transcriptional repressors. We selected *Vibrio natriegens* as the host organism due to its superior growth rate and exceptional protein expression capabilities, successfully establishing sensor engineering within this framework. Additionally, codon optimization has been employed to enhance protein expression. This study aims to develop synthetic platforms for naringenin and sakuranetin. Furthermore, we have made preliminary advancements in the development of biosensors capable of endogenous detection, thereby facilitating directed evolution pathways for substances that are difficult to detect, thus highlighting the significant potential of *V. natriegens* and biosensor technology.

Keywords: Directed evolution; Biosensor; *Vibrio natriegens*; TtgR; Chalcone synthase

1.Introduction

1.1 Sakuranetin and Naringenin

Sakuranetin (4,5-dihydroxy-7-methoxyflavanone) is a compound that belongs to dihydroflavonoid. In plants, it is synthesized from the precursor substance naringenin and exists as a phenolic plant antitoxin. In recent years, sakuranetin have been widely reported for its multiple pharmacological benefits, including antioxidant, anti-inflammatory, anti-mycobacterial, anti-viral, anti-fungal, anti-Leishmania protozoan, anti-trypanosomal, glucose uptake stimulation, neuroprotective, antiretroviral generation, and antitumor properties.

Today, most flavonoids are synthesized either by direct extraction from plants or by chemical methods. Therefore, direct extraction is limited by plant growth conditions and unstable biomass supply for continuous production of flavonoids. Moreover, the two existing methods of extraction are costly and cannot reach a large scale. Biosynthesis, as a highly potential production method, has been reported in articles using chassis such as *Escherichia coli* and *Saccharomyces cerevisiae* as model organisms, but the yield is still a long way from reaching the requirements of industrial production. As reported, this pathway is limited by the condensation reaction of p-coumaroyl coenzyme A with three molecules of malonyl-CoA, which is catalyzed by chalcone synthase. Optimization of the enzymatic profile of chalcone synthase becomes critical for improving yield. When considering how to optimized the pathway to maximize the output, we use several methods in our design: to use the enzyme with high catalytic activity, and to increase the protein expression level by using codon optimized coding sequence and high-efficient promoters.

1.2 *Vibrio natriegens*

V. natriegens is a Gram-negative marine bacterium with a fast growth rate of 15.61 ± 0.18 min in the logarithmic phase, compared with 27.23 ± 0.69 min for *E. coli* cells. Compared with common chassis organisms such as yeast and *E. coli*, *V. natriegens* has a faster growth rate, a broader substrate spectrum, superior substrate uptake and an abundant number of ribosomes in the cell, which contributes to its excellent exogenous protein expression ability, so *V. natriegens* is a new type of chassis cell that has been developed in the field of biotechnology and synthetic biology in recent years. Based on its excellent biological properties, a variety of

natural products and proteins, such as L-DOPA[1], melanin[2], c-type cytochromes[3] and other substances, have been efficiently biosynthesized in *V. natriegens*. Moreover, *Vibrio* exhibits robust applicability to commonly used synthetic biology parts. The synthetic biology tools for the *V. natriegens* are maturing, which shows further promises.

1.3 Biosensor

In the directed evolution process, high-throughput screening is the main bottleneck. Biosensors offer the benefits of high sensitivity, real-time monitoring, and efficiency properties. Previous flavonoid product assays have relied on high performance liquid chromatography (HPLC), biosensor also eliminates the need for purification of cultures as well as enrichment. A variety of promoters can further alter the sensitivity of biosensors by modulating protein expression. In this study, we choose to express the TtgABC-TtgR system from *Pseudomonas putida* DOT-T1E in *V. natriegens*, and it can reflect the product concentration in real time and efficiently.

2.Design and results

2.1 Construction of Plasmids for Biosynthesis of Naringenin and Sakuranetin

Large-scale synthesis of flavonoids relies on the complement of precursors, and the selection of a suitable precursor as a substrate is critical considering the economic cost. The main synthetic pathways commonly used for naringenin are the phenylalanine pathway and the tyrosine pathway. In the phenylalanine pathway, the step from the substrate phenylalanine to p-coumaric acid is catalyzed by phenylalanine ammonia lyase (PAL), cinnamic acid 4-hydroxylase (C4H) and 4-coumaroyl CoA ligase (4CL). In contrast, in the tyrosine pathway, tyrosine ammonia lyase (TAL) catalyzes the direct conversion of tyrosine to 4-coumarate, bypassing the C4H intermediate. For heterologous protein expression systems, going through as few steps as possible is the best way to minimize the accumulation of intermediates and their associated effects. Another barrier was that C4H, which is a membrane-bound P450 monooxygenase, could not be functionally expressed in bacteria. Therefore, we ultimately chose the tyrosine pathway for naringenin production. To synthesize sakuranetin, O-methyltransferase 3 (OMT3) is needed. The overall pathway including enzymes, substrates and

products are shown in Figure 1.

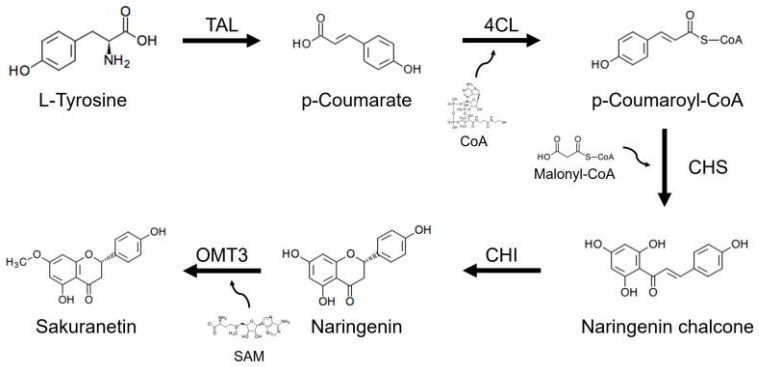

Figure 1. Overall biosynthesis pathway of naringenin and sakuranetin used in this study. Figures of different compounds were cited from KEGG.

The enzymes engaged in this study come from various species, having a relatively higher catalytic activity compared to their isoenzyme. The enzymes' origin and reference are shown in Table 1, and their sequence are shown in Supplementary information Table S1.

Table 1. Enzymes' names, origins and their NCBI Reference Sequence

Enzyme	Origin	Reference
TAL	*Aspergillus lentulus*	NCBI Reference Sequence: XM_033561525.1
4CL	*Arabidopsis thaliana*	GenBank: MT134263.1
CHS	*Eubacterium ramulus*	PDB: 4D06_A
CHI	*Erigeron breviscapus*	GenBank: AED02599.1
OMT3	*Perilla frutescens*	GenBank: MT909556.1

To increase the protein expression level, we decided to employ T7 promoter to drive the expression of the enzymes. Considering that there's T7 RNA polymerase coding sequence in *V. natriegens*' genome and the relatively more complex method to modify its genome, we constructed a T7 RNAP expression cassette. When choosing the plasmid backbone, we found that *V. natriegens* can only recognize a few replication origins like f1-ori and p15A-ori, and plasmids with pMB1/pBR322/pUC origin cannot replicate in it. Due to plasmid incompatibility, we chose pETDuet-1 and pACYCDuet-1 as the backbone. The two plasmids have different origins of replication so they can co-exist in *V. natriegens*. What's more, both of them have two

expression cassettes driven by T7 RNAP and LacI/LacO so the expression of protein can be controlled by Isopropyl-beta-D-thiogalactopyranoside (IPTG). We replaced the first expression cassette on pETDuet-1 with T7 RNAP cassette driven by lac promoter, and inserted the 5 genes into remaining 3 expression cassettes. Ultimately, we successfully constructed the pETDuet-1-4CL-TAL and pACYCDuet-1-OMT3-CHI-CHS plasmids utilizing the AlTAL gene, At4CL gene, PfOMT3, EbCHI gene, and ErCHS gene.

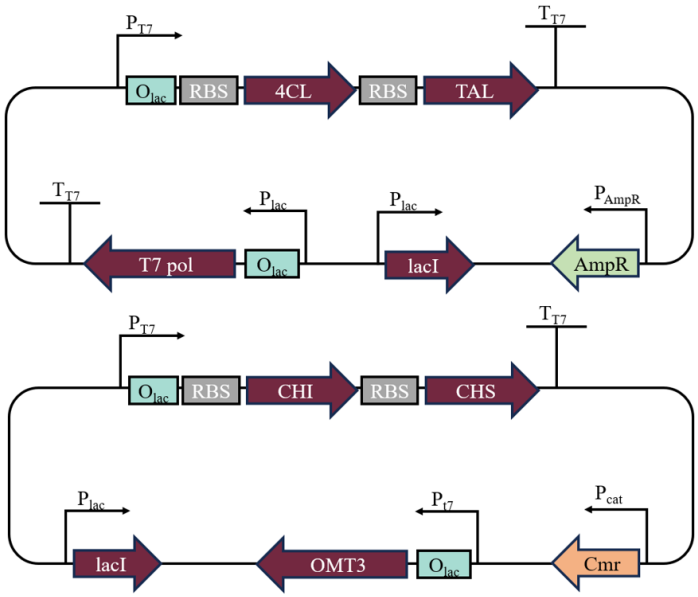

Figure 2. The structure of pACYCDuet-1-TAL-4CL and pETDuet-1-OMT3-CHI-CHS

2.2 Codon Optimization of CHI and CHS

We choose Genetic Algorithm (GA) for codon optimization.GA is an optimization algorithm that simulates natural selection and genetic mechanisms. It belongs to the category of evolutionary computation and aims to find the optimal solution to the problem by simulating the process of biological evolution. The basic idea of genetic algorithm originates from Darwin's theory of natural selection.

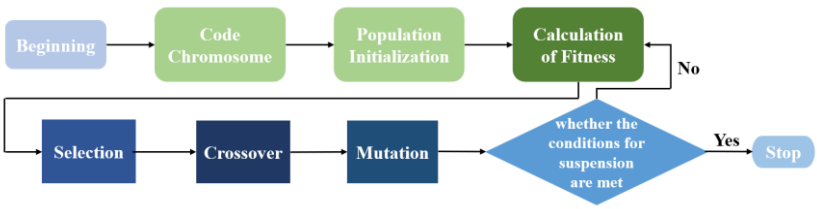

Figure 3. Basic flowchart of genetic algorithm(GA)

Individual codon usage (ICU) is considered to be one of the most critical factors affecting the efficiency of mRNA translation. But previous studies have also reported significant effects of codon pair usage, also known as codon context (CC), on protein expression levels. Therefore, we chose ICU fitness and CC fitness as conditions. And the resultant sequence is ICU-/CC-optimal one.

In the analysis of codon usage patterns, significant differences in ICU and CC distributions between highly expressed genes and other genes confirmed the importance of using highly expressed genes to characterize preferred codons. We downloaded the 2000 most highly expressed genes of *V. natriegens* (ATCC 14048) from the GenBank as reference sequences and counted the codon usage frequency and codon pair occurrence frequency of these genes. After the number of iterations of the algorithm is reached, the optimized sequence is outputted.

2.3 Designing Biosensor for Detection

The TtgABC-TtgR system from *Pseudomonas putida* DOT-T1E encodes an efflux pump involved in resistance to antibiotics and aromatic compounds whose expression is activated by such molecules via the transcriptional repressor TtgR.

TtgR repressors can undergo conformational changes by binding to a variety of flavonoids and antibiotics, and de-repression occurs upon release from the homologous operon. Considering that the chemical formula and three-dimensional structure of naringenin chalcone and phloretin are very similar (differing only by one carbon-carbon double bond), and after molecular docking simulation, we attempt to construct this system in *V. natriegens* for real-time detection of intracellular naringenin/naringenin chalcone concentration. This biosensor is mainly used in two directions. One is for high-throughput screening to guide directed evolution, and the other is for yield detection of final pathway synthesis products.

We chose the codon-optimized green fluorescent protein (GFP) gene as the reporter gene, and fused it to the promoter of the TTG operon. In the absence of naringenin or naringenin chalcone, the transcriptional regulator TtgR binds to the pseudo-palindromic DNA sequence of the operon to repress its expression. Upon binding of the inducer to the active binding site of TtgR, the repressor is shed from the binding site and the downstream gene GFP initiates expression.

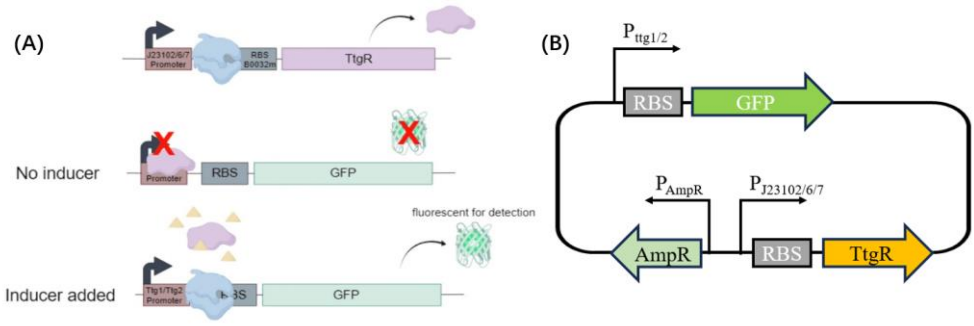

Figure 4. (A) The principles of the TtgR repressor; (B) The structure of pACYC177-TtgR-GFP

For the high-throughput screening of directed evolution, we constructed multiple plasmids with different strengths of constitutive promoters controlling TtgR Repressor to obtain biosensors with appropriate sensitivity and more suitable for directed evolution. Meanwhile, to ensure that the system can work properly in *V. natriegens*, we constructed plasmid versions of two different TtgR repressor operons. As mentioned in Xiong's article, the wild-type-derived TtgABC operon part could not successfully initiate the expression of red fluorescent protein (RFP) in *E. coli*[7]. Based on the common promoter sequence of *E. coli*, a T/A mutation was introduced at the tenth base site of this operon to obtain the TtgABC2 sequence, which is proven to successfully initiate downstream gene expression.

We detect the fluorescence intensity by enzyme-labeled instrument, and monitor the cellular OD_{600} at the same time. Finally, we homogenize the data by fluorescence intensity/cell density, so as to evaluate the correspondence between different inducer concentrations and fluorescence intensity.

It is noteworthy that naringenin itself, as a flavonoid, has an inhibitory effect on the growth of *V. natriegens*. This was found through the comparative graphs of growth curves, which may likewise affect the effect of protein expression.

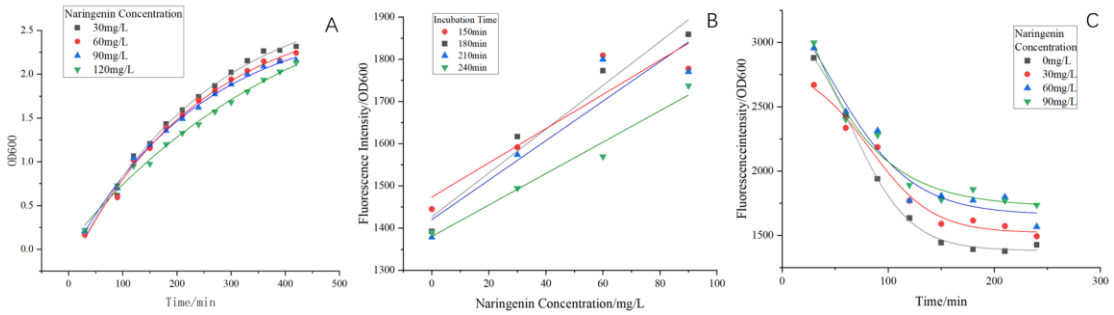

Figure 5. Verifying the effectivity of TtgR-GFP sensor. A. Growth curve of V.natriegens in different concentration of naringenin; B. Ratio of GFP fluorescence:OD600 (relative fluorescence intensity) under different concentration of naringenin; C. Relative fluorescence intensity under different concentration of naringenin.(B: 150min fitted curve R^2 :0.775; 180min fitted curve R^2 :0.943; 210min fitted curve R^2 :0.782; 240min fitted curve R^2 :0.958)

The GFP showed a certain fluorescence intensity without the addition of inducers, resulting in a certain fluorescence leakage. In the available data obtained, we get the expected, linear growth curve over a range of inducer concentrations. According to the fitted curve, the effect caused by background fluorescence gradually decreased with time, and the homogenized fluorescence intensity showed a relatively stable linear enhancement relationship with naringenin concentration after the incubation time reached 150 min. Although their homogenized values do not show a clear multiplicative relationship, their stable linear relationship proves that they can be used to assist High-through screening for directed evolution.

Moreover, we combined protein structure analysis, simulation, and molecular docking, trying to shorten the directed evolutionary pathway by changing the detection substrate to naringenin chalcone, introducing single mutations for more application possibilities.

We performed molecular docking simulations of TtgR and naringenin chalcone, and then selected the optimal docking possibilities by comparing the fractions of the combined binding energies. The white numbers represent binding energy, and we chose the models with the highest score. The crucial sites are D172, E78, S77 and C137, which are partly different from the crucial sites in the binding mode of phloretin to TtgR. Experimental validation of whether TtgR can detect naringenin chalcone is in progress.

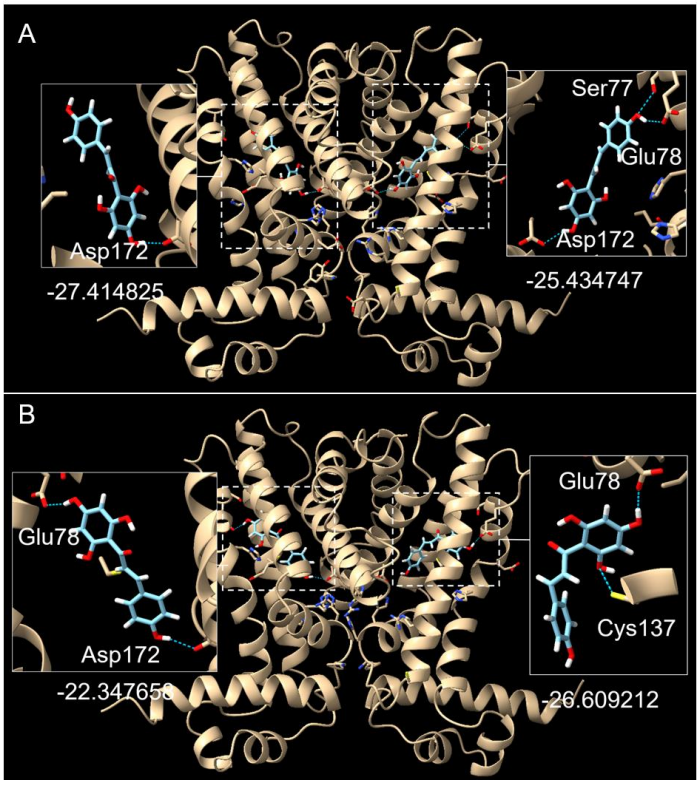

Figure 6. A. Molecular docking simulation TtgR with naringenin chalcone

For a more sensitive biosensor, we refer to the binding mode of phloretin to TtgR to guide the construction of naringenin chalcone-targeted sensors. The site 176 is a high affinity one for phloretin. In *Pseudomonas putida* strain KT2440, which has a low tolerance to toxic chemicals, the corresponding amino acid is glycine. Studies have shown that G176R can effectively enhance the binding ability to phloretin.

For pathway product detection, with reference to the yield concentration achievable in existing production (1+g/L), we want a sensor that could be applied in large-scale production, with lower sensitivity and higher requirements for inducer concentration. Based on the current research, we select to mutate site N110, site L66 and site V96. Each site individually showed a clear decrease in affinity for naringenin and phloretin, which demonstrated a better rate of DNA release only at higher concentration. At the same time, those mutations had a higher affinity for naringenin than for phloretin.

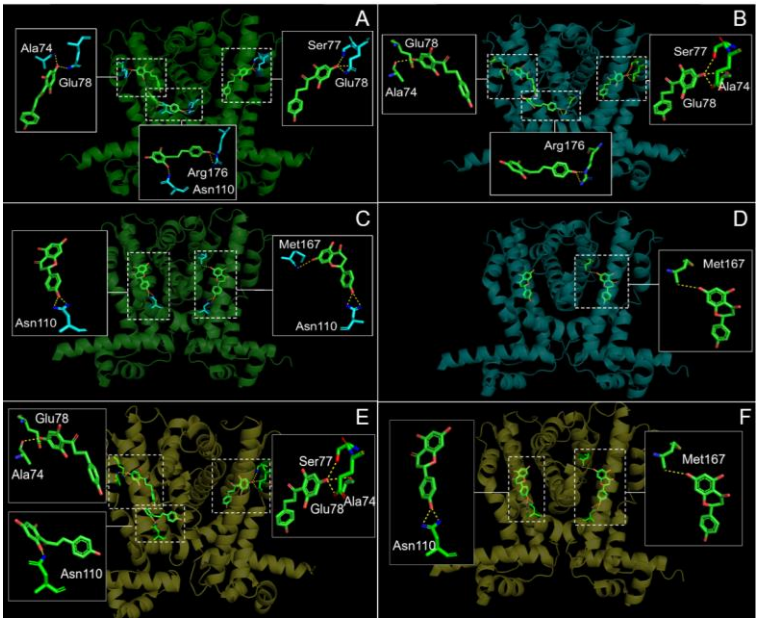

Figure 7. A. Molecular docking modeling of TtgR mutant(G176R) with phloretin; B. Molecular docking modeling of TtgR mutant(L66A, V96A, N110A, G176R) with phloretin; C. Molecular docking modeling of TtgR mutant(G176R) with naringenin; D. Molecular docking modeling of TtgR mutant(L66A, V96A, N110A, G176R) with naringenin; E. Molecular docking modeling of wild-type TtgR with phloretin; F. Molecular docking modeling of wild-type TtgR with naringenin;

We are still working on the transformation of a plasmid containing only the GFP fluorescent protein gene to investigate the basal expression level of fluorescent proteins under the ttg1/ttg2 promoter in the absence of the repressor. A variety of plasmids with mutations are being constructed.

2.4 Directed Evolution of CHS

The designed and constructed intracellular biosensor can reflect the amount of product by fluorescence intensity. Although CHS can catalyze p-coumaroyl coenzyme A in one step to obtain detectable naringenin chalcone. However, the price of p-coumaroyl coenzyme A is relatively high, so we decided to use its precursor, p-coumaric acid, as a substrate. Then we inserted the mutant sequence of CHS and CHI gene into the constructed pACYCDuet-1-4CL to obtain a series of plasmids for targeted evolutionary effect detection. Ultimately, the TtgR-GFP biosensor will be applied to detect the concentration of naringenin in vivo, which can reflex the result of direct evolution of CHS. As shown in Figure 8, we will transform both plasmids into *V. natriegens*. With the combination of 4CL, CHS and CHI, the bacterium can produce naringenin from p-coumalic acid. The catalyst ability varies among different CHS

mutants, resulting in various naringenin concentration in different clones. Compared to HPLC, biosensor can reflex real-time naringenin concentration and can save time from preparing the samples for HPLC, making it a useful tool in directed evolution.

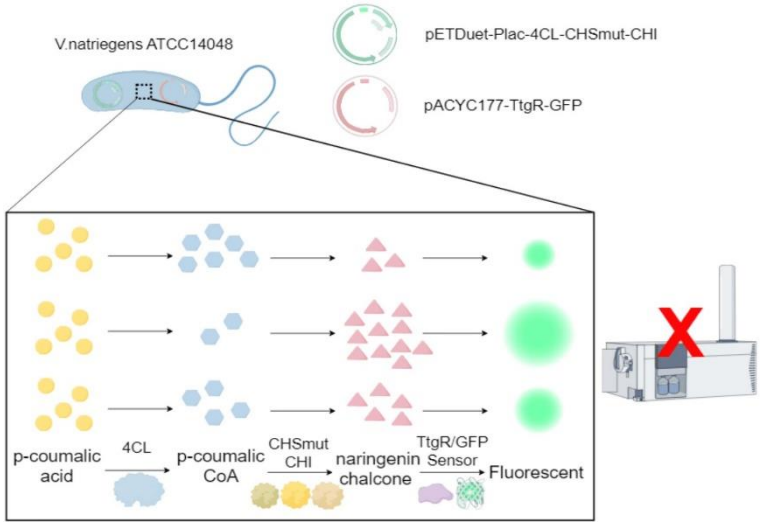

Figure 8. Dual plasmid system for in high-throughput screening of *V. natriegens*

During our research, we found that the mechanism of byproduct formation in CHS catalysis may involve lactonization-type ring closure, or it may be due to the release of catalytic intermediates from the catalytic site. Therefore, having a more stable protein structure may be a practical way to minimize byproduct formation. Xiang's[13] article mentions that T145M substitution leads to the formation of new hydrophobic bonds and possesses stronger stability values. Although the EbCHS we chose and the mutants originated from different organisms, we selected the mutation A82M and the neighboring N83K after the protein sequence comparison. Meanwhile, based on the increased naringenin abundance and yield in the article, we performed the mutations V37D, M96L, K219E, A296Q, and A297G, and verified the effect of the single-point mutation F153V, as well as the effect of multiple mutation combinations.

3.Discussion

Our results suggest that the addition of the inducer released TtgR to a certain extent, but the sensitivity was not strong enough. And the resources used for growth are more than used for protein expression, resulting in a faster increase in cell density than the increase in

fluorescent protein expression. We are trying to investigate the optimal conditions for intracellular protein expression by varying the carbon-to-nitrogen ratio and adjusting the culture temperature.

The design and successful construction of a high-throughput screening system combining directed evolution with a biosensor allowed efficient screening of CHS mutants. This method significantly improved screening efficiency compared to traditional approaches, facilitating the rapid identification of effective CHS variants. However, longer mutation validation pathways increase the complexity of mutation library construction. Also, more intermediates may mean less efficient production of the product. This places higher demands on the sensitivity of the sensor.

Although progress was made in enzyme expression, there is still a need to balance the metabolic flux within the synthetic pathway. Adjusting the expression levels of different enzymes, avoiding intermediate accumulation, could further enhance production yield. The current TtgR-based biosensor demonstrated limited sensitivity in detecting naringenin, affecting the effectiveness of high-throughput screening. Future work could involve protein engineering to enhance TtgR specificity for naringenin or explore other more sensitive biosensors to improve screening efficiency. *V.natriegens* showed a tendency to prioritize growth over target protein expression, which may limit product yield. Optimizing medium composition, adjusting induction conditions, or using metabolic engineering to redirect resources towards target product synthesis could further improve production efficiency.

This study provided an effective way of improving CHS through an innovative high-throughput screening method combining directed evolution and biosensors. This approach not only enhanced screening efficiency but also showcased the potential of *V. natriegens* as a chassis organism for industrial production. However, achieving more efficient and scalable production will require further research and optimization in metabolic pathway regulation, biosensor performance, and cultivation strategies.

4.Methods

4.1 Strains and Materials

In this study, *E.coli* Trelief5α (Tsingke) was used for plasmid construction and amplification. *V.natriegens* strain ATCC14048 (Mingzhou Bio) was chosen as the chassis strain for constructing microbial cell factories for flavonoids. *E. coli* BL21(DE3) (Tsingke) and *V. natriegens* strain ATCC14048 (Mingzhou Bio) are used for verification of the function of the biosensor. Chemical reagents were purchased from Shanghai Sangon and Aladdin. The primers were obtained from Anhui General Biol.

4.2 Plasmids Construction and Point Mutation

The genes 4CL, TAL, CHS, CHI and OMT3 were synthesized by Shanghai Sangon. The gene coding T7 RNA polymerase was obtained from the laboratory's frozen dock. The plasmid backbone pETDuet-1 was obtained from Shanghai Sangon and the plasmid pACYCDuet-1, pACYC177 were purchased from Miaoling Biology. All the plasmids were constructed using the homologous recombination method.

4CL was cloned into the second expression cassette of pETDuet-1. To drive the expression of gene under T7 promoter, we constructed a T7 RNA polymerase expression cassette with lac promoter, lac operator and CAP binding site. Then we replace the first expression cassette of the plasmid backbone with the newly built T7-RNAP expression cassette. After adding RBS, TAL was inserted into the second expression cassette at the downstream of 4CL.

OMT3 was inserted into *Pst*I site in the first expression cassette of pACYCDuet-1. Then we amplified CHS, CHI and linearized pACYCDuet-1-OMT3 plasmid by PCR, and finally inserted CHI and CHS into the second expression cassette using multi-fragments homologous recombination.

The genes GFP, TtgR and J23102/6/7 promoters were synthesized by Anhui General Biol. The GFP expression cassette with Ttg2 promoter was synthesized and used to replace the KanR on pACYC177. Variant TtgR expression cassette with J23102/6/7 promoter and B003m RBS is then inserted into the plasmid backbone.

4.3 *Vibrio natriegens* transformation

Before the transformation, pick up a single colony of *V. natriegens* on the LB plate and

transfer into 20 mL LB liquid medium and culture overnight under the condition of 30 ℃, 200 rpm. Pipette 0.5 mL culture and transfer into 50 mL BHIv2 (BHI medium, 2M NaCl, 231 mM $MgCl_2$, 42 mM KCl) liquid media in a 250 mL flask, then incubated under 37 ℃, 200 rpm until OD600=0.5. Collect 10 mL culture, chill on ice for 15 min and centrifugate at 3000 rcf under 4 ℃ for 5 min. Remove the supernatant and resuspend the bacteria in 10 mL transformation buffer (680 mM sucrose, 7 mM K_2HPO_4), then centrifugate at 3000 rcf under 4 ℃ for 5 min. Repeat the process twice to wash the remained salts completely. Then resuspend the bacteria with 300 μL cold transformation buffer.

When performing transformation, add 1.25 μg plasmid into 50 μL competent cell on ice. Transfer the cell-DNA suspension to a chilled electroporation cuvette with a 1mm gap size. Cells were electroporated using the following parameters: 1200 V, 25 μF, 200 Ω. Then immediately recover the cell with 500 μL 37 ℃ recover medium (BHIv2 with 680 mM sucrose), transfer into a conical tube and incubate at 37 ℃ under 50 rpm for 1 h. Plate the recovery culture on LB plate with appropriate antibiotic and incubate at 37 ℃ for 14 h.

4.4 GFP sensing

Strains that have been successfully electroporation with the desired plasmid and colonized for PCR validation were cultured under the condition of 200 rpm, 37° overnight. Pipette 0.5 mL culture and transfer into 50 mL BHIv2 liquid media in a 250 mL flask, then incubated under 37 ℃, 200 rpm until OD_{600}=0.5.Take multiple 1 ml samples and transfer to 24-well plates. Add naringenin or naringenin chalcone reservoirs of graded concentration. Incubate on shaker for graded time. Measure the OD_{600} and spike the sample in 96-well black plates. Measure the fluorescence under microplate reader.

4.5 Genetic Algorithm

Multiple iterations are required in this algorithm. In each iteration we do the following steps. Firstly, we calculate the ICU fitness and CC fitness calculation. p_0^k is the frequency of the kth codon in the reference sequence and p_1^k is the frequency of the kth codon in the current individual; q_0^k is the frequency of the kth codon pair in the reference sequence, and q_1^k is the frequency of the kth codon pair in the current individual.

$$\psi_{ICU} = -\frac{\sum_{k=1}^{64} |p_0^k - p_1^k|}{64}$$

$$\psi_{CC} = -\frac{\sum_{k=1}^{3904} |q_0^k - q_1^k|}{3904}$$

Then we calculate the individual fitness. Each round first initializes the fitness of each individual to 0. Then all pairs of individuals in the population are traversed, and for each pair, there are (ψ_{ICU1}, ψ_{CC2}) and (ψ_{ICU2}, ψ_{CC2}). Adaptation is evaluated according to the following rules:

$$if\ \psi_{ICU1} > \psi_{ICU2}\ and\ \psi_{CC1} \geq \psi_{CC2},\ sequence\ 1\ dominates\ sequence\ 2$$

$$if\ \psi_{ICU1} \geq \psi_{ICU2}\ and\ \psi_{CC1} > \psi_{CC2},\ sequence\ 1\ dominates\ sequence\ 2$$

$$if\ \psi_{ICU1} < \psi_{ICU2}\ and\ \psi_{CC1} \leq \psi_{CC2},\ sequence\ 2\ dominates\ sequence\ 1$$

$$if\ \psi_{ICU1} \leq \psi_{ICU2}\ and\ \psi_{CC1} < \psi_{CC2},\ sequence\ 2\ dominates\ sequence\ 1$$

Whenever a particular sequence is found to be dominated by another sequence, the domination rank of the former sequence is lowered. Finally, we preform the fitness normalization and selection. For the selection in the algorithm, we use the roulette wheel method. Since the algorithm can only be applied to cases where the fitness value is positive, we normalize the fitness, after which we apply the corresponding value to the roulette wheel method, and all the individuals of the original population and the new population are sorted according to the fitness, and the part of the individuals with the lowest fitness is discarded to ensure that the number of individuals in the population remains unchanged. When the maximum number of iterations is reached, the algorithm ends and the sequence with the highest fitness is output.

4.6 Molecular Docking

The PDB documents of TtgR in complex with naringenin and phloretin are cited from RCSB PDB (2UXU and 2UXI)[5] .We use Pymol to visualize molecular docking. After removing water molecules, the binding hydrogen bonds between TtgR and small ligands are displayed, and the name and sequence number of hydrogen-bonded residues are labeled. The same work is also done after introducing four single mutations.

The prediction of the docking of TtgR and naringenin chalcone comes from Swiss dock. We download the SDF file of naringenin-chalcone from pubchem, use OpenBabel to convert it to mol2 format and upload it and the pdb file of TtgR to Swiss dock to predict the binding situation. After the computing is finished , we use Chimera X to display the prediction result,

select two results that the ligand separately binds to the two subunits of the dimer with the lowest intermolecular energy and show hydrogen bonds between ligands and residues of TtgR.

5.Acknowledgements

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6.Author Contribution

L. Luo, T. Kong, J. Sun and F. Yang designed the plasmids. S. Fu and Y. Fan performed codon optimization. S. Zhang, N. Jiang, T. Kong performed directed evolution on CHS and TtgR. All the members carried out the experiments. L. Luo, S. Fu, S. Zhang, Y. Ma, H. Gao, X. Bao, F. Yang and J. Dong wrote the report. F. Yang, J. Dong, S. Zhang and Y. Wang contributed to the experimental operation teaching of laboratory members. Z. Xu built the web page. T. Kong managed the laboratory. J. Hong guided the design and experimentation of this project.

7.Conflict of interest

All the authors declare no conflict of interest.

8.Supplementary information

Materials, genes' sequences, primers and protocols are shown in supplementary information.

9.References

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