Multi-level flux optimization at the key regulatory node for enhanced production of naringenin using acetate in engineered Escherichia coli

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Research Article

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Abstract

Background

Microbial production of naringenin has received much attention due to its pharmaceutical applicability and potential as a key molecular scaffold for various flavonoids. In particular, the oxaloacetate (OAA) node is a key regulatory node for the naringenin biosynthesis from acetate, acting as a critical linkage that reroutes tricarboxylic acid (TCA) cycle intermediates via anaplerosis of the glyoxylate cycle to the naringenin biosynthetic pathway. In this context, to efficiently produce naringenin from acetate, it is crucial to precisely regulate the carbon flux of the OAA-PEP regulatory node through appropriate \( pckA \) expression control, as the excessive overexpression of \( pckA \) can cause the extensive loss of OAA and metabolic imbalance. However, considering the crucial impact of \( pckA \) on naringenin biosynthesis, the conventional strategy of single-level gene expression is limited in its ability to cover the large and balanced solution space, which could result in suboptimal naringenin production.

Results

This study conducted multi-layer fine-tuning of \( pckA \) expression for the precise exploration of optimal naringenin production from acetate in the large and balanced solution space. Specifically, a combinatorial expression library was generated at both transcriptional and translational levels through promoters with different strengths and rationally designed 5′-UTR variants with discrete translation efficiency. Additionally, we identified the effect of multi-level regulation of \( pckA \) expression by validating the correlation between PCK activity and naringenin production. As a result, the flux-optimized strain demonstrated a significant increase in naringenin production, with a 49.8-fold increase (and a 73.8-fold increase in naringenin yield on acetate) compared to the unoptimized strain, producing 122.12 mg/L naringenin with 20.65 mg naringenin/g acetate, which is a comparable result against those from conventional substrates.

Conclusions

Collectively, we demonstrated the significance of multi-level expression control at the key regulatory node in the metabolic pathway, covering the large and balanced solution space for precise flux rebalancing. This study proposes a platform strain for the biosynthesis of various flavonoids that can be derived from naringenin using acetate.

Background

Naringenin is a value-added chemical that can be broadly applied for pharmaceutical uses with its properties of oxygen radical elimination, anti-inflammatory, and antiviral [1–3]. In addition, naringenin is a key scaffold chemical for the biosynthesis of various flavonoids [4, 5]. Traditionally, naringenin has been extracted from natural plants, which has limitations such as low yield from plants, high requirements for solvents, intricate purification process, and high-cost issues [5–7]. Therefore, biosynthesis of naringenin through microbial fermentation via heterologous production in \textit{Escherichia coli} and \textit{Saccharomyces}
cerevisiae has been studied as a breakthrough with advances in metabolic engineering and synthetic biology. To achieve economically feasible microbial production of naringenin, utilizing a low-cost and plentiful feedstock is an important issue. In this context, acetate has been proposed as a highly promising feedstock for industrial uses, as it can be sufficiently obtained from inexpensive natural sources such as lignocellulosic biomass and carbon dioxide at reasonable costs [8–10]. Additionally, acetate can be derived from organic wastes and industrial syngas, showing its potential as an eco-friendly resource [11, 12]. Therefore, utilizing acetate for the biosynthesis of naringenin would be an efficient strategy for achieving an economical bioproduction process.

For the bioconversion of acetate to naringenin, improving acetyl-CoA availability is necessary for a sufficient supply of malonyl-CoA for the naringenin biosynthetic pathway [13–15]. Indeed, the previous study has identified key regulatory nodes for naringenin biosynthesis from acetate, indicating enhanced naringenin production with increased acetyl-CoA availability through the flux redistribution between cell growth and naringenin production [16]. Particularly, the upregulation of phosphoenolpyruvate carboxykinase (PCK) at the oxaloacetate (OAA) node acted as a critical linkage that reroute tricarboxylic acid (TCA) cycle intermediates via anaplerosis of the glyoxylate cycle to the naringenin biosynthetic pathway [17, 18].

To maximize naringenin production from acetate, it is required to precisely optimize the carbon flux at the OAA node through appropriate expression levels of pckA (encoding PCK). From this perspective, gene expression control for efficient microbial conversion has been conducted in previous studies by altering transcription efficiency or translation efficiency [19–23]. However, these strategies for optimization of gene expression have mainly regulated the single-level stage, which could lead to a suboptimal point with limited coverage of large and balanced solution space [24, 25]. Specifically, in the OAA node, excessive overexpression of pckA can cause the extensive loss of OAA, resulting in a significant metabolic imbalance and decreased cell growth and naringenin production. Thus, the expression of pckA must be precisely regulated to achieve optimized naringenin production due to its considerable influence on flux distribution. In this context, at the branch point between cell growth and production, such as the OAA node, optimum flux rebalancing through a more advanced fine-tuning of the gene expression beyond the single expression stage would be an effective strategy for enhanced naringenin production from acetate.

In this study, we conducted complex and multi-level fine-tuning of pckA gene expression in both transcriptional and translational levels to explore a large and balanced solution space at the OAA node (Fig. 1). To precisely tune pckA expression, we constructed systematic mutant libraries using promoters with varying strengths and 5'-UTR variants that could generate different expression levels. Specifically, we rationally designed 5'-UTRs with predicted translation efficiencies using the UTR Library Designer to establish a more balanced expression range beyond transcriptional variation. With the constructed variants of pckA expression, we examined the capacity of these pckA expression variants for naringenin production and the enzymatic activity of PCK, investigating the optimal expression of pckA at the OAA node under multi-level flux rebalancing. Consequently, the flux-optimized strain produced 122.12 mg/L of naringenin (a yield of 20.65 mg naringenin/g acetate) under optimized culture conditions, showing a
49.84-fold increase over that of the unoptimized strain, which reaches a competitive result to those from conventional substrates (Additional file 1: Table S1). Collectively, our study highlights the importance of multi-level optimization at both transcriptional and translational levels to maximize product formation, presenting an effective metabolic engineering strategy for the biosynthesis of naringenin and its various flavonoid derivatives using acetate.

**Results And Discussion**

**Systematic regulation of pckA expression for improved production of naringenin from acetate**

For multi-level optimization of naringenin biosynthesis from acetate, we took advantage of BNIAP variants as base strains which were constructed for the naringenin biosynthesis from acetate in our previous study [16]. While expression regulation of pckA, a key regulatory gene, was attempted at transcriptional levels in the study, we anticipated that advanced fine-tuning of the gene expression for further exploration of precise and balanced solution space would be important for the optimum flux rebalancing, considering the significant effect of PckA activity on flux distribution in the naringenin biosynthetic pathway. To this end, based on our previous examination of pckA expression variance under different promoters and the corresponding naringenin production [16], we investigated the solution space of PCK activity between the synthetic promoter BBa_J23115 and BBa_J23113 for the further optimization of naringenin production (Fig. 2a). Accordingly, we generated mutant libraries of gene expression using rationally designed 5′-UTR variants for the optimum flux at the OAA regulatory node. Specifically, we constructed five variants of pckA expression with different 5′-UTR sequences with computationally predicted translation efficiency for the promoters through the UTR Library Designer [26]: BNIAP115U1, BNIAP115U2, BNIAP115U3, BNIAP115U4, and BNIAP115U5 for the promoter BBa_J23115; BNIAP109U1, BNIAP109U2, BNIAP109U3, NIAP109U4, and BNIAP109U5 for the promoter BBa_J23113 (from high to low translation efficiency, in order).

We evaluated the naringenin production capacity of all pckA variants using acetate as a carbon source (Fig. 2a). Among them, BNIAP115U4 exhibited the highest naringenin production of 78.47 mg/L and specific production of 91.50 mg/g DCW, which is a 32.1-fold and 61.41-fold increase over those of the base strain BN, which harbors the recombinant pathway of naringenin biosynthesis (Fig. 2a and 2b). Additionally, naringenin yield on acetate in the BNIAP115U4 strain (13.87 mg naringenin/g acetate) was 49.5-fold higher than that of the BN strain, indicating a significant enhancement in the conversion of acetate to naringenin due to multi-level precise tuning of pckA expression (Additional file 1: Table 2). Overall, we demonstrated that the multi-layer regulation of pckA beyond the single-level stage was important for optimizing the naringenin production from acetate, which lead to the optimum flux redistribution at the key branch node.

**Validation of effects of multi-level precise tuning of pckA on the enhancement of naringenin production**

To enhance naringenin production from acetate, increasing the availability of acetyl-CoA to ensure sufficient a supply of malonyl-CoA is a significant challenge [13–15]. In this context, rerouting TCA cycle
intermediates through anaplerosis of the glyoxylate cycle to the naringenin biosynthesis has been shown to be important [16]. Accordingly, upregulating \textit{pckA} to facilitate carbon flux from OAA to PEP would be an effective strategy for maximizing naringenin production. However, excessive \textit{pckA} overexpression could cause significant OAA loss, leading to a critical metabolic imbalance and decreased naringenin production. Therefore, to explore the optimal point of flux redistribution at the OAA node, we generated \textit{pckA} mutants with multi-layer variations of their expression and evaluated their naringenin production. To thoroughly validate the effects of precise and balanced expression control on naringenin biosynthesis, we examined the PCK activities of the mutants. Specifically, we verified the correlation of PCK activity and naringenin production over the \textit{pckA} mutants whose expression was complexly regulated at both transcriptional and translational levels.

For examination of the \textit{pckA} expression levels of the constructed expression library, we conducted PCK activity assays of the variants in the mutant library designed with different promoter strengths and computed translation efficiencies of the 5′-UTRs (Fig. 3). The results indicated that the balanced expression range was successfully generated within a specific exploration range, and naringenin production capacity enhanced with increased PCK activity. However, as we speculated that extensive loss of the OAA could lead to detrimental effects on the cells, naringenin production decreased when \textit{pckA} was overexpressed. Furthermore, remarkably, the difference in PCK activity due to translational regulation beyond the transcriptional level led to differences in naringenin production capacity, indicating that a sophisticated exploration for the appropriate \textit{pckA} expression was essential for flux optimization. Collectively, systematic engineering for the proper \textit{pckA} expression at both transcriptional and translational levels was an effective strategy for maximizing naringenin biosynthesis. Furthermore, we successfully demonstrated that precise regulation in the branch node of cell growth and production was highly important for precise flux rebalancing, leading to efficient naringenin production from acetate.

\textbf{Optimization of cultivation conditions for enhanced naringenin production}

For further improvement of naringenin production, we conducted optimization of culture conditions for the BNIAP115U4 strain by regulating the expression of the \textit{pckA} expression at multiple levels. In this study, 4CL, CHS, and CHI, the key heterologous enzymes, were expressed under the T7 promoters using IPTG as an inducer for the naringenin biosynthesis. Since the optimum induction point and induction level were associated with the allocation of cellular resources for protein biosynthesis [27–29], the optimum culture condition must be specified experimentally for a particular system, which may vary depending on the host cells and carbon substrates for naringenin production [30,31]. Therefore, to achieve maximized naringenin biosynthesis, we investigated the optimal IPTG induction condition for the heterologous key enzymes to balance the distribution of cellular components between cell growth and naringenin production.

To achieve this, we evaluated the naringenin production capacity of the BNIAP115U4 according to different induction points and IPTG concentrations. First, in the range of OD\textsubscript{600} 0.6 to 3.0 as induction points, the BNIAP115U4 exhibited the highest naringenin titer at the induction point of OD\textsubscript{600} 1.0 (Fig. 4a),
indicating the importance of the proper transition point from cell growth to production. Additionally, the BNIAP115U4 showed the highest naringenin production with the addition of 0.05 mM IPTG in the concentration range of 0.001 to 1.0, emphasizing the appropriate heterologous expression of 4CL, CHS, and CHI for the optimized production of naringenin from acetate. Notably, compared to BNIAP109U1 with only transcriptional optimization of pckA expression under the optimized culture condition, BNIAP115U4 with pckA expression regulated at both the transcriptional and translational levels had a 32% increase in naringenin production (Additional file 1: Fig. S1). Consequently, we achieved the highest naringenin titer of 122.12 mg/L with naringenin yield (20.65 mg naringenin/g acetate and 0.664 g naringenin/g p-coumaric acid) under the optimized culture conditions at the induction point of OD$_{600}$ 1.0 with the addition of 0.05 mM IPTG (Fig. 4b), which is competitive with the results from previous studies on conventional substrates such as glucose or glycerol (Additional file 1: Table S1).

Conclusions

In this study, our goal was to optimize naringenin biosynthesis from acetate by systematically engineering the expression of pckA. The efficient microbial production of naringenin from acetate requires enhancing acetyl-CoA availability for a sufficient supply of malonyl-CoA, a precursor of naringenin biosynthesis. From this perspective, upregulating pckA, which converts OAA to PEP, for rewiring TCA cycle intermediates to the naringenin production pathway can increase acetyl-CoA availability. However, extensive OAA loss with over-expressed PCK can lead to critical metabolic imbalance, decreasing naringenin production. Accordingly, the exploration of the appropriate pckA expression level is essential for the optimization of naringenin biosynthesis. To this end, in the study, we performed multi-layer fine-tuning of the pckA expression. Using a computational design through UTR Library Designer based on synthetic promoters, we constructed a precise and balanced mutant library of pckA expression regulated by both transcriptional and translational levels. The resulting strain, BNIAP115U4, exhibited the highest naringenin production capacity of 122.12 mg/L (with a yield of 20.65 mg naringenin/g acetate) under the optimized condition, a 49.8-fold increase of production capacity (with a 73.8-fold increase of naringenin yield on acetate) over that by the unoptimized strain. Furthermore, we validated the impact of multi-level optimization of pckA on naringenin biosynthesis, evaluating the correlation between PCK activity and naringenin production. Collectively, this study demonstrated the importance of systematic engineering with multi-layer gene expression control at the key regulatory node involved in the flux redistribution between cell growth and naringenin production, achieving a platform strain for the production of various flavonoids derived from naringenin using acetate.

Methods

Reagents and primers

Plasmid and genomic DNA were purified using GeneAll® Plasmid SV kit and GeneAll® Exgene™ Cell SV kit (GeneAll Biotechnology, Seoul, Korea), respectively. Q5® High-Fidelity DNA Polymerase, restriction endonucleases, and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA, USA). T4
Polynucleotide Kinase and EmeraldAmp® PCR Master Mix were purchased from Takara Bio Inc. (Shiga, Japan). Oligonucleotides were synthesized by Cosmogenetech (Seoul, Korea). Other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless specified.

**Construction of bacterial strains and plasmids**

Bacterial strains and plasmids used in this study are listed in Additional file 1: Table S4. *E. coli* Mach1-T1® was used to proliferate all plasmids for cloning experiments and naringenin production was performed in BL21 Star™ (DE3) and its derivatives. For the naringenin production, transcriptionally optimized pFlavo$^{opt}$, containing 4-coumaroyl-CoA ligase (4CL) from *Arabidopsis thaliana*, chalcone synthase (CHS) from *Petunia hybrida*, and chalcone isomerase (CHI) from *Citrus maxima*, was obtained from a previous study [31], and other genes were derived from the genomic DNA of *E. coli* BL21 Star™ (DE3). The genetic information on constitutive promoters (Anderson promoter series) and terminator (BBa_B1001) was acquired from the Registry of Standard Biological Parts (http://parts.igem.org). 5′-untranslated regions (5′-UTRs) with specific predicted expression levels were designed using the UTR Library Designer [26].

Restriction enzyme digestion and ligation were used for the construction of plasmids pACYCA and pACYCAP115. Specifically, *acs* and *pckA* genes were amplified from the genomic DNA of *E. coli* BL21 Star™ (DE3) via two sequential PCRs to introduce the constitutive promoter and synthetic 5′-UTR. For the insertion of *acs*, the vector fragment was obtained from pACYCduet-1, as a template, using Vector_acs_speI_F/Vector_acs_notI_R. The purified PCR fragments of *acs* and its vector were digested using SpeI and NotI and assembled, resulting in pACYCA. Similarly, the vector fragment was amplified from pACYCA as a template, using Vector_pckA_kpnI_F/Vector_pckA_notI_R, for the insertion of *pckA*. The resulting fragment and the *pckA* PCR fragment were digested using KpnI and NotI, and assembled to construct pACYCAP115. Plasmids with all variants of *pckA* (pACYCAP104, pACYCAP106, pACYCAP109, pACYCAP113, pACYCAP103, pACYCAP115U2, pACYCAP115U3, pACYCAP115U4, pACYCAP115U5, pACYCAP109U2, pACYCAP109U3, pACYCAP109U4, pACYCAP109U5) were constructed through blunt-end cloning using the forward primers with corresponding constitutive promoter sequence and the reverse primer (*pckA*_blunt_R). Deletion of the chromosomal *iclR* gene was conducted by the Lambda-Red recombination method using the plasmids pKD46 and pCP20 [32].

**Culture conditions for naringenin production**

*E. coli* strains were cultivated in Andrew’s Magic Medium (AMM) containing 100 mL of 10× MOPS mixture [33], 5.0 g/L K$_2$HPO$_4$, 3.5 g/L KH$_2$PO$_4$, 3.5 g/L (NH$_4$)$_2$HPO$_4$, 2 g/L casamino acid, 0.1 mL of 5 g/L thiamine-HCl, and 0.1 mL of 1 M CaCl$_2$ supplemented with 10 g/L NaOH-neutralized acetate (pH 7.0) as a
carbon source. Antibiotics (100 mg/L ampicillin and 34 mg/L chloramphenicol) were added to the medium for plasmid maintenance.

For naringenin production, single colonies of each strain were inoculated in 15-mL test tubes containing 3 mL fresh AMM and incubated overnight at 37 °C with continuous shaking (200 rpm). Thereafter, saturated broths were inoculated in 300-mL Erlenmeyer flasks containing 25 mL fresh medium, at an OD$_{600}$ of 0.1, and incubated to reach an OD$_{600}$ of 1.0. Refreshed culture broths were re-inoculated into 25 mL fresh medium with OD$_{600}$ of 0.1 and incubated at a culture temperature of 37 °C, with agitation at 200 rpm. When culture broths reached an OD$_{600}$ of 1.0, 200 mg/L $p$-coumaric acid and 1 mM isopropyl $\beta$-d-thiogalactopyranoside (IPTG) for the induction of 4cl, chs, and chi genes were added to the broth, lowering the temperature to 30 °C after induction. All experiments were performed in biological triplicates. OD$_{600}$ of the broths was recorded using a UV-1700 spectrophotometer (Shimadzu Co., Kyoto, Japan) and the pH was adjusted to 6.8-7.1, with a 5 M HCl solution, using an Orion™ 8103BN ROSS™ pH meter (Thermo Fisher Scientific, Waltham, MA, USA). Culture samples were periodically collected and stored at -80 °C for further analysis.

**Enzyme activity assay**

The promoter strength variants were cultivated until each variant had the same OD$_{600}$ value, then the cell pellets were harvested by centrifugation at 15,814×g for 10 min at 4 °C. Cell lysates were prepared through the addition of 0.1 mM phosphate buffer solution (pH 8.0) to the cell pellets and disruption by the sonication (Q125 Sonicator, Qsonica, CT, USA). Enzyme activity was assayed according to the previous study with minor modifications [34]. The reaction mixture containing 4 mM ATP, 4 mM MgCl$_2$, 4 mM oxaloacetate, and 0.1 mM phosphate buffer solution was added to cell lysates up to 180 µl, then enzyme reactions were performed for 60 min at 30 °C. The consumption of oxaloacetate was measured using an Aminex HPX-87H column (Bio-Rad Laboratories, Richmond, CA, USA) and Shodex RI-101 detector (Shodex, Klokkerfaldet, Denmark), using 5 mM H$_2$SO$_4$ as the mobile phase at a flow rate of 0.6 mL/min at 14 °C [34].

**Metabolites analysis**

Ultimate 3000 high-performance liquid chromatography system (Dionex, Sunnyvale, CA, USA) was used to analyze the metabolites contained in culture broths. For the detection of naringenin production and $p$-coumaric acid consumption, the culture broth and an equal amount of absolute ethanol were mixed and centrifuged for 10 min at 13000 rpm. Thereafter, the supernatant was analyzed with an Acclaim 120 C18 column (Dionex) and a UV–vis diode array detector. Acetonitrile and water, each containing 0.1% formic acid, were used as the mobile phase, at a flow rate of 1 mL/min, using the following multi-gradient flow program: 10-40% acetonitrile for 0-10 min and 40-60% acetonitrile for 10-15 min with absorbance detection at 280 nm. Acetate consumption was identified with Aminex HPX-87H column (Bio-Rad Laboratories, Richmond, CA, USA) and Shodex RI-101 detector (Shodex, Klokkerfaldet, Denmark), using 5 mM H$_2$SO$_4$ as the mobile phase at a flow rate of 0.6 mL/min at 14 °C.
Abbreviations

**TCA cycle**: Tricarboxylic acid cycle

**OAA**: Oxaloacetate

**PEP**: Phosphoenolpyruvate

**E. coli** *Escherichia coli*

**4CL**: 4-coumaroyl-CoA ligase

**CHS**: Chalcone synthase

**CHI**: Chalcone isomerase

**PCK**: Phosphoenolpyruvate carboxykinase

**IPTG**: Isopropyl β-d-thiogalactopyranoside

**5’-UTRs**: 5′-untranslated regions

**DCW**: Dry cell weight

Declarations

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Availability of data and materials**

All data generated or analyzed during this study are included in this published article and its supplementary information files.

**Competing interests**

The authors declare that they have no competing interests.
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**Authors’ contributions**

D.H.K., H.G.H., and G.Y.J. designed the project. D.H.K. and H.G.H. performed experiments. D.H.K., H.G.H., and G.Y.J. analyzed the data and D.H.K., H.G.H., and G.Y.J. wrote the manuscript. G.Y.J. supervised the project. All authors approved the final version of the manuscript.

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**References**


**Figures**
Figure 1

Schematic diagram of systematic engineering of *pckA* at both transcriptional and translational levels. PEP, phosphoenolpyruvate; 5′-UTR, 5′-untranslated region; TCA cycle, tricarboxylic acid cycle.

Figure 2

Comparison of the constructed multi-level *pckA* expression variants in (a) the production of naringenin and (b) the fermentation profile of the BNIAP115U4 strain. Flask cultures were performed for 48h in biological triplicates. Error bars indicate the standard deviations of biological triplicates.
Effect of multi-level fine-tuning of *pckA* for the production of naringenin. The bar plot illustrates normalized PCK enzyme activities for various mutants with expression levels modulated by distinct promoters and engineered 5′-UTRs. Multi-level regulated *pckA* mutants are ordered in the x-axis as an activity-increasing row. The naringenin production capacity of each variant is marked as a scatter plot. Error bars indicate the standard deviations of biological triplicates.

**Figure 3**
Figure 4

Culture condition optimization of the BNIAP115U4 strain. (a) Evaluation of naringenin production capacity under different conditions of IPTG concentrations and induction times. (b) Fermentation profile of the BNIAP115U4 strain under optimized culture conditions, such as induction time at OD$_{600}$ of 1.0 with the addition of 0.05 mM IPTG. Flask cultures were performed for 48 h in biological triplicates. Error bars indicate the standard deviations of biological triplicates. BNIAP115U4 strain refers to E. coli BL21 Star™(DE3) with heterologous expression of essential enzymes for naringenin production, $acs$ overexpression, $iclR$ knockout, and $pckA$ upregulation under constitutive promoter BBa_J23115 and synUTR$_{pck4}$; IPTG, isopropyl $\beta$-d-thiogalactopyranoside.

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