Development of Artificial Riboswitches for Monitoring of Naringenin In Vivo

Sungho Jang,†,‡ Sungyeon Jang,†,‡ Yu Xiu,‡,§,∥ Taek Jin Kang,† Sang-Hyeup Lee,⊗ and Gyoo Yeol Jung*,†,¶

†Department of Chemical Engineering, Pohang University of Science and Technology, 77 Cheongam-ro, Nam-gu, Pohang, Gyeongbuk 37673, Korea
‡Department of Chemical and Biological Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York 12180, United States
§State Key Laboratory of Chemical Resource Engineering, Beijing University of Chemical Technology, Beijing 100029, China
∥Beijing Key Laboratory of Bioactive Substances and Functional Food, Beijing Union University, Beijing 100191, China
¶Department of Chemical and Biochemical Engineering, Dongguk University, 30 Pildong-ro 1-gil, Jung-gu, Seoul, 04620, Korea
⊗Department of Life Chemistry, Catholic University of Daegu, Hayang-ro 13-13, Hayang-eup, Gyeongsan, Gyeongbuk 38430, Korea
VDepartment of Biological Sciences, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York 12180, United States
¶School of Interdisciplinary Bioscience and Bioengineering, Pohang University of Science and Technology, 77 Cheongam-ro, Nam-gu, Pohang, Gyeongbuk 37673, Korea

Supporting Information

ABSTRACT: Microbial strains are considered promising hosts for production of flavonoids because of their rapid growth rate and suitability for large-scale manufacturing. However, productivity and titer of current recombinant strains still do not meet the requirements of industrial processes. Genetically encoded biosensors have been applied for high-throughput screening or dynamic regulation of biosynthetic pathways for enhancing the performance of microbial strains that produce valuable chemicals. Currently, few protein sensor-regulators for flavonoids exist. Unlike the protein-based trans-regulating controllers, riboswitches can respond to their ligands faster and eliminate off-target effects. Here, we developed artificial riboswitches that activate gene expression in response to naringenin, an important flavonoid. RNA aptamers for naringenin were developed using SELEX and cloned upstream of a dual selectable marker gene to construct a riboswitch library. Two in vivo selection routes were applied separately to the library by supplementing naringenin at two different concentrations during enrichments to modulate the operational ranges of the riboswitches. The selected riboswitches were responsive to naringenin and activated gene expression up to 2.91-fold. Operational ranges of the riboswitches were distinguished on the basis of their selection route. Additionally, the specificity of the riboswitches was assessed, and their applicability as dynamic regulators was confirmed. Collectively, the naringenin riboswitches reported in this work will be valuable tools in metabolic engineering of microorganisms for the production of flavonoids.

KEYWORDS: naringenin, riboswitch, aptamer, riboswitch tuning, sensor-regulator, flavonoid

Flavonoid is a class of secondary metabolites that are produced from plants or fungi.1 These molecules are now in the limelight owing to their beneficial effects on human health, including antioxidative, anti-inflammatory, and anti-cancer activities.2 However, flavonoids cannot be produced economically using traditional methods such as extraction from natural sources because of low productivity and high costs.3 Contrary to the traditional approach, microbial production has characteristics such as rapid growth rate and scalability, which are suitable for the large-scale economic production of flavonoids.4,5 Although several studies have investigated the development of microbial strains for flavonoid production through metabolic engineering, the recombinant strains should be improved further for industrial production.

Recently, genetically encoded biosensors have been used for improving the titer, yield, and productivity of microbial cell factories.6 Biosensors consist of protein or RNA and control gene expression in a ligand-dependent manner.7 Owing to their ability of regulating gene expression as a function of target

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metabolite concentration, biosensors were applied to high throughput screening, real-time monitoring, and dynamic control of metabolic flux.\textsuperscript{6–10} For flavonoids, bacteria-derived transcription factors were utilized to construct genetic circuits that control the expression of fluorescent proteins in response to quercetin,\textsuperscript{11} kaempferol,\textsuperscript{11} and naringenin.\textsuperscript{11,12} However, protein-based systems require the expression of transcription factors, which imposes an unnecessary metabolic burden on cells.\textsuperscript{13–15} In addition, their trans-regulatory mechanism of action often delays response and causes off-target effects.

Unlike protein-based sensors, RNA-based biosensors cause less metabolic burden on cells.\textsuperscript{16,18} Moreover, some classes of RNA biosensors act in cis, making the response faster\textsuperscript{20} and eliminating any undesired off-target effect. These cis-acting RNA biosensors are called riboswitches, and they are generally located in the 5\textsuperscript{′}-portion of mRNAs, the transcription or translation of which is controlled by the riboswitches.\textsuperscript{21} Riboswitches control the expression of genes by changing their conformation upon binding of specific molecules,\textsuperscript{22} and they respond to various target ligands such as cofactors, amino acids, and metal ions with high affinity and specificity, which are comparable to those of protein-based controllers.\textsuperscript{16,23} Natural or artificial riboswitches have been engineered to understand molecular interactions and structural dynamics,\textsuperscript{24} comprehend cellular metabolism,\textsuperscript{25} validate antimicrobial targets,\textsuperscript{26} and fabricate genetic devices that do not interfere with endogenous regulatory mechanisms.\textsuperscript{27,28} Especially, owing to the ability of riboswitches to measure intracellular metabolite concentration, they have been utilized for screening or selection of highly productive bacterial strains.\textsuperscript{29,30} Even though a wide range of molecules can be detected by natural riboswitches, and several artificial riboswitches have been developed so far, riboswitch that regulates gene expression in response to flavonoids has not yet been reported. Therefore, development of artificial riboswitches that respond to flavonoids can facilitate the engineering of highly productive recombinant strains for industrial production of these molecules. For example, a riboswitch that can detect the range of flavonoid concentrations that can be currently produced by microbial strains could be utilized to dynamically control the expression of pathway genes in response to the flavonoid. A riboswitch that can discriminate higher concentration of flavonoid, meanwhile, could be employed to screen or select producer strains that are improved compared to the current strains.

Here, we report the development of artificial riboswitches that respond to a flavonoid molecule, naringenin. Naringenin has several health benefits, and it serves as a central scaffold molecule for the synthesis of various flavonoids.\textsuperscript{1} First, we selected RNA aptamers for naringenin using an in vitro selection method. Then, we constructed a riboswitch plasmid library by directly cloning the heterogeneous pool of aptamers obtained from the in vitro selection without a comprehensive analysis of individual aptamers. Two selection routes were designed and applied to the \textit{Escherichia coli} population transformed with the riboswitch plasmid library for the evolution of functional riboswitches in vivo. Specifically, we modulated the concentration of naringenin and the sequence of negative and positive selections in different selection routes. As a result, six riboswitches, three from each route, were successfully generated. Overall workflow of in vitro and in vivo selections utilized in this study is illustrated in Figure S1. Interestingly, the evolved riboswitches showed a clear difference in operational ranges, and the half maximal effective concentrations (EC\textsubscript{50}) differed depending on the selection route used. In addition, we validated the high specificity of the riboswitches to naringenin, which renders them useful for dynamic regulation of flavonoid biosynthesis pathways. The naringenin riboswitches with the various characteristics obtained in this study can be used as valuable tools for detecting and monitoring intracellular or extracellular naringenin.

**RESULTS AND DISCUSSION**

**Selection of RNA Aptamers for Naringenin.** Artificial RNA aptamers that bind to naringenin were selected in vitro. The pool of DNAs that contained a randomized sequence region between two primer binding sites was transcribed using T7 RNA polymerase (Figure 1A). We set the length of the randomized sequence to 50 nucleotides to maximize the probability of discovering naringenin aptamers,\textsuperscript{31} whereas the size of the pool was minimized as much as possible. In the initial DNA library, about 0.903 × \textsuperscript{10\textsuperscript{15}} molecules were contained, which can provide sufficient sequence diversity for a successful selection.\textsuperscript{32} Naringenin was immobilized on the
epoxy-activated sepharose 6B to construct the affinity matrix for in vitro selection (Figure 1B). The epoxide group of the resin and the hydroxyl groups of naringenin can react to form an ether linkage at high pH. The efficiency of the reaction was estimated by measuring the absorbance of the naringenin-containing coupling solution at 320 nm before and after the coupling reaction (Figure S2A). The concentration of naringenin was reduced by 26.875 mM (Figure S2B), which suggests that more than half of the epoxide groups on the resin were coupled to naringenin.

Next, the RNA pool was enriched using the affinity matrix. The RNA pool and the affinity matrix were mixed together and incubated for the binding reaction to occur. The RNA fraction that bound to the affinity matrix was recovered, reverse transcribed, and polymerase chain reaction (PCR)-amplified to prepare the pool for the next round. The percentage of the bound fraction overshot at round 2, reaching 10.9%, but it was stabilized again at round 3 and remained lower than 2% until round 5 (Figure 1C). This fluctuation has been occasionally observed in several SELEX experiments, and this pattern seems to vary depending on the library and the target molecule. After round 6, the percentage abruptly increased to 21.2%. We performed a negative selection at round 7 using an intact matrix without naringenin to remove the RNAs that bind to the matrix rather than to naringenin. After one more round of positive selection, the RNA population was enriched with the naringenin binders by 26.1%. We stopped the selection process at this stage to maintain the diversity of the RNA pool.

**In Vivo Selection of Naringenin Riboswitches.** Naringenin riboswitches were selected in vivo, and their operational ranges were engineered by modulating the selection conditions. First, the riboswitch library was constructed by cloning the aptamer pool upstream of tetA-sGFP, a dual selectable marker gene in a backbone plasmid (Figure 2A). We used the entire enriched RNA pool directly, instead of using specific, well-characterized, and tight-binding aptamers, to maximize the probability of obtaining aptamers with switching functionality in vivo. Unlike a previous report that cloned a heterogeneous pool of aptamers directly upstream of the Kozak sequence,40 we inserted an additional 10-nucleotide randomized linker sequence between the aptamer and the ribosome binding site (RBS), since this linker was shown to affect the activity of the artificial riboswitches. A library of $10^7$–$10^8$ colonies was successfully prepared using this design (data not shown).

Next, we optimized the selection conditions to select naringenin riboswitches with appropriate operational ranges. First, the extent of selection pressure was optimized for efficient selection. *E. coli* strains that harbored variants of the backbone plasmid with diverse expression levels of the selection marker were prepared. Various concentrations of tetracycline or NiCl$_2$ were supplemented to the culture broths of the strains and their growth rates were measured (Figure S3). We decided to use 50 µg/mL tetracycline and 10 mM NiCl$_2$ for positive and negative selection, respectively, because the BBa_J23113 promoter was used for the construction of the riboswitch library (see Methods). Then, selection routes were designed to evolve the riboswitches with the operational ranges suitable for quantification of naringenin in recombinant producer strains. The operational range of a riboswitch is a range of the ligand concentration where the riboswitch can discriminate changes in the ligand concentration. The naringenin concentrations for positive selection were selected based on the naringenin concentration produced by the recombinant strains. Currently, most recombinant *E. coli* strains produce naringenin lower than 100 mg/L when glucose is used as a substrate. Since the naringenin riboswitches should be able to screen or select improved strains compared to the current strains, we added 100 or 200 mg/L naringenin for positive selection in the “Mid” or “High” selection routes, respectively (Figure S1). Negative selections were performed prior to the positive selection with low concentrations of naringenin to exclude the riboswitch variants that activate gene expression at low concentration. We hypothesized that the riboswitches from the “High” route would be activated at a higher naringenin concentration compared to the riboswitches from the “Mid” route, because they underwent a negative selection with 100 mg/L naringenin instead of a positive selection that the “Mid” riboswitches had experienced at the same naringenin concentration.

We selected six colonies, three from each route, after two cycles of each selection route were applied to the riboswitch library (Figure S4). Naringenin-dependent activation of gene expression was tested using the fluorescence from the reporter gene, tetA-sGFP. Every selected riboswitch showed approximately 1.93–2.91 fold increase in fluorescence upon addition of 200 mg/L naringenin to the growth medium (Figure 2B). While there was no significant difference between the fold activations of the Mid and High riboswitches ($p = 0.251$, Mann–Whitney rank sum test), the expression levels of the High riboswitches were lower than that of the Mid riboswitches when either riboswitches were fully induced ($p < 0.001$) or not induced ($p = 0.006$). This trend probably reflects the nature of the selection routes; more negative selections were performed in the High route than in the Mid route. Sequencing revealed that the features such as primer binding sites, randomized linker, and the RBS of the riboswitches were retained as designed (Table S1). Intriguingly, two riboswitches from...
different selection routes, Mid2 and High2, contained the same aptamer sequence, the only difference being the randomized linker region.

**Dose–Responses Differed Depending on the Selection Route.** The dose–response of the riboswitches was investigated by measuring fluorescence in the presence of naringenin at various concentrations. A dramatic contrast was observed between the dose–response curves of the riboswitches obtained from different routes (Figure 3A, S5). For example, the EC$_{50}$ of High1 (324.6 mg/L) was 12.6-fold higher than that of Mid1 (25.7 mg/L) (Figure 3B). All other riboswitches showed similar trends in the EC$_{50}$ values.

**Figure 3.** Dose–response curves and EC$_{50}$ of the naringenin riboswitches. (A) Comparison of the dose–response curves of Mid2 and High2 riboswitches. Fold activations of fluorescence in the presence of naringenin at various concentrations were fitted to the sigmoidal dose–response equation. filled circle: Mid2; empty triangle: High2; solid lines: dose–response curves of the riboswitches. Error bars indicate standard deviations of three biological replicates. (B) EC$_{50}$ of the riboswitches. EC$_{50}$ was defined as the concentration of naringenin at which the riboswitch exhibits 50% response compared to the full-range of the response. EC$_{50}$ values were obtained from the fitting of the fold activation to log (naringenin). Error bars indicate standard errors.

**Figure 4.** Response of the riboswitches to flavonoids. (A) Specific fluorescence was measured using FACS in the presence of 0.2 mM flavonoids, and the values were normalized to the fluorescence when 0.2 mM naringenin was supplemented. Carbon numbering of the flavonoids is shown on the upper left. Naringenin and eriodictyol are differed by only −H and −OH attached to carbon at 3′ position. Naringenin, eriodictyol, and flavanone contain a single bond between the carbons at 2 and 3 positions, while luteolin and apigenin contain a double bond between the carbons at the same positions. The result of a hierarchical clustering of the flavonoids is shown on the left of the heat map. (B) Demonstration of the specificity of Mid3 riboswitch in a producer strain. The background strain was transformed with either Mid3 alone or Mid3 along with pFlavoopt. The plasmid pFlavoopt contained a flavonoid pathway (4CL–CHS–CHI) that is capable of catalyzing reactions from p-coumaric acid (p-C) to naringenin and from caffeic acid (CA) to eriodictyol. Specific fluorescence was measured when either p-coumaric acid or caffeic acid were added at 0.6 mM to the culture medium. Error bars indicate the standard deviation from biological triplicates.
depending on the selection route used. This result demonstrates that the operational ranges of riboswitches can be engineered by applying different selection conditions. Different from common approaches for screening and selection of artificial riboswitches, additional negative selection was performed in the presence of low amount of ligand (10 mg/L naringenin) in this study. Additionally, positive selections were performed at two different ligand concentrations to select the riboswitches with distinct quantitative behaviors. The simplicity and accessibility of this approach seem remarkable considering that previous studies required intensive analyses and optimizations based on detailed knowledge of biochemistry, regulatory mechanism, and evolutionary relationship of the subject riboswitches. Interestingly, dose–response curves of Mid2 and High2 were clearly distinct from each other even though they differed only in the linker sequences. This result underlines the importance of the linker region for engineering the dose–responses of riboswitches, in addition to the binding affinity of aptamers.\(^{42-45}\) Computational prediction using Mfold\(^{49}\) revealed that the secondary structures of two riboswitches were highly different from each other (Figure S6). This simulation utilized the linker-RBS-CDS (30 nt) region, the only difference between the riboswitches upon binding of naringenin to the aptamer. Secondary structure of RNA is known to significantly influence the efficiency of mRNA degradation.\(^{49}\) Therefore, the degradation rates of the riboswitches might have been differed owing to their structures. The difference in degradation rate could be a reason for the difference in EC\(_{50}\) between Mid2 and High2, since the degradation rate of mRNA is one of the factors that determines EC\(_{50}\) of riboswitches.\(^{47}\) Since the Mid riboswitches can detect the range of naringenin concentration that can be produced by current microbial strains, they could be utilized to dynamically control the expression of pathway genes in response to naringenin. The High riboswitches, meanwhile, could be employed to screen or select producer strains that are improved compared to the current strains, because they can discriminate higher concentration of naringenin (>100 mg/L).

Response of the Naringenin Riboswitches to Other Flavanoids. Naringenin is not only a valuable molecule in itself but also a precursor to various flavonoids. Genetic controllers responsive to metabolic intermediates have been exploited to improve metabolite production by dynamically regulating metabolic pathways.\(^{7,50}\) To be utilized as a dynamic regulator, the naringenin riboswitches should be able to discriminate between naringenin and other derived flavonoids. Therefore, we examined the specificity of the riboswitches using four additional flavonoids. Eriodictyol and flavanone belong to the same class of flavonoids as naringenin, and they are produced from different precursors. Apigenin or luteolin can be synthesized from naringenin or eriodictyol, respectively, using a single enzyme.

Fluorescence intensities in the presence of flavonoids were normalized using the fluorescence observed in the presence of naringenin to calculate the fold activations (Figure 4A). Hierarchical clustering of the flavonoids shows that the riboswitches are generally more responsive to naringenin, eriodictyol, and flavanone than luteolin and apigenin, with the exception of Mid2 which was activated by all tested flavonoids. This tendency could result from the dissimilarity between the molecular structures of the flavonoids. Naringenin, eriodictyol, and flavanone contain a single bond between the carbon atoms at positions 2 and 3; however, luteolin and apigenin contain a double bond between the carbon atoms at the same positions, which substantially alters their structure. Generally, high specificity of a biosensor is desired as many structurally related molecules are present inside a cell. In this regard, Mid3 would be the best riboswitch for metabolic engineering, because it specifically responds to naringenin, not to the molecules with similar structures. However, naringenin and eriodictyol are produced from different substrates, and, therefore, the two molecules generally have less chance to be in the same cell. Therefore, Mid1, Mid2, High1, and High2 could be utilized for screening or selection of eriodictyol production when only the substrate for this metabolite is fed to the producer strain. Additionally, two riboswitches with similar EC\(_{50}\) values, Mid2 (32.48 mg/L) and Mid3 (37.39 mg/L), showed clearly different specificities toward the flavonoids tested. Therefore, sensitivity of a riboswitch does not ensure the riboswitch to be highly specific only to its target.

The ability of a naringenin riboswitch to discriminate the production of different flavonoids was demonstrated in a recombinant producer strain. Mid3 riboswitch was transformed to the producer strain\(^{51}\) which contained a flavonoid pathway consists of 4-coumaroyl-CoA ligase (4CL), chalcone synthase (CHS), and chalcone isomerase (CHI) from plants. This strain was able to synthesize naringenin or eriodictyol using p-coumaric acid or caffeic acid as a substrate, respectively. We supplemented the substrates to culture medium and measured specific fluorescence. The specific fluorescence was about 4 times higher upon addition of 0.6 mM p-coumaric acid to the medium compared to when the substrate was not added to the medium (Figure 4B). On the contrary, the addition of caffeic acid to the medium did not cause noticeable change of the specific fluorescence compared to the negative control (no substrate added). This result demonstrates the specificity of Mid3 riboswitch to discriminate the production of different flavonoids in the producer strain.

Interestingly, Mid2 and High2, which contain the same aptamer, showed apparent discrepancy in fold activations when luteolin and apigenin were used as inducers. The specificity of riboswitches largely originates from the specificity of the aptamers. However, this result implies that the linker sequence, which was known to control only the activation of riboswitches, can also affect the specificity of the riboswitches. Although the role of these sequences in determining specificity needs to be investigated in future, the potential of the linker as a tunable city was demonstrated in this study. Overall, the riboswitches were able to distinguish between different flavonoids, and they could be used for dynamic regulation of metabolic pathways in addition to their potential use as platforms for screening and selection of improved producer strains.

**Methods**

Preparation of a Naringenin-Coupled Matrix. Naringenin (Sigma-Aldrich, St. Louis, MO) was immobilized on the epoxy-activated sepharose 6B (GE Healthcare Life Sciences, Pittsburgh, PA) according the manufacturer’s instruction. The KCl–NaOH buffer (pH 13) was used for coupling reaction because the reaction between the epoxide group of the solid support and the hydroxyl groups of naringenin requires high pH.\(^{11}\) The naringenin stock solution (1 M) in dimethylformamide (DMF) was diluted in the coupling buffer at a final concentration of 0.1 M to prepare the coupling solution. The swollen and washed matrix was mixed with the coupling...
solution and incubated for 16 h at 30 °C with gentle shaking. Coupling of naringenin was confirmed by measuring the absorbance at 320 nm (Figure S2). The uncoupled epoxide groups were blocked using 1 M ethanolamine.

**In Vitro Selection of the RNA Aptamers.** The RNA aptamers that bind to naringenin were selected *in vitro*. The DNA template was prepared by PCR amplification of 1.5 nmol of SELEX_Template oligonucleotide using Phusion DNA polymerase (New England Biolabs, Ipswich, MA) with SELEX_F and SELEX_R primers (Table S2). The amplified DNA pool was transcribed using T7 RNA polymerase (New England Biolabs). The RNA was purified by polyacrylamide gel electrophoresis after the treatment with DNase I (Takara Bio Inc., Nojihigashi, Japan).

Three nmoles of the RNA pool was dissolved in 1 mL of a selection buffer (50 mM Tris-HCl, 100 mM KCl, pH 7.4) and refolded by heat denaturation at 95 °C for 10 min followed by cooling to room temperature for 20 min. MgCl₂ was added to the RNA solution at a final concentration of 10 mM, and the solution was stabilized at room temperature for 15 min. The RNA pool was mixed with 1 mL of the naringenin-coupled matrix in a polypropylene column (Qiagen, Hilden, Germany) and incubated overnight at room temperature with gentle rotation. Selection round 7 utilized the uncoupled matrix without naringenin for a negative selection.

The RNA-matrix mixture was filtered through the in-column filter of the polypropylene column, and the remaining matrix was extensively washed using 10 column volumes of a washing buffer (50 mM Tris-HCl, 100 mM KCl, 10 mM MgCl₂, pH 7.4). Then, the matrix was mixed with 2 mL of an elution buffer (25 mM Tris-HCl, 300 mM NaCl, 5 mM EDTA, 4 M urea, pH 7.4) and heated to 95 °C to elute RNAs. The flow-through from the elution steps were precipitated using ethanol, and the RNA concentrations were measured using a UV-1700 spectrophotometer (Shimadzu, Kyoto, Japan) at 260 nm.

The eluted RNA was reverse transcribed using SuperScriptIII (Invitrogen, Carlsbad, CA) and SELEX_R. This cDNA was amplified by PCR using SELEX_RT_T7 and SELEX_R as primers. The amplified DNA served as a template for the preparation of RNA for the next selection round. The washed fraction from round 7 was used instead of the eluted fraction for round 8.

**Construction of the Riboswitch Library.** The riboswitch plasmid library (pNRNRibo_library, Table S3) was constructed by cloning the enriched RNA pool into a backbone plasmid (pRibo_NC). First, the plasmid was created by PCR amplification of pTrpRibo with the primer set (Ph_J23113_NC_R and Ph_NC_F), which were phosphorylated at their 5'-end and contained BsaI restriction sites. The PCR product was ligated using the Quick Ligation kit (New England Biolabs). The ligated DNA was transformed into E. coli Mach1-T1K cells.

The PCR amplified plasmid and the PCR-amplified aptamer pool were digested using BsaI and ligated using the Quick Ligation kit. The ligated product was transformed into E. coli DH10B competent cells, and the plasmids were purified using a mini-preparation method. The riboswitch plasmid library was transformed into E. coli W3110 cells.

**In Vivo Selection of the Naringenin Riboswitches.** The riboswitch library was enriched by employing the dual selectable marker (tetA). The marker, tetA, encodes a tetracycline/H⁺ antiporter which pumps out tetracycline while facilitates the uptake of nickel ion. Therefore, cells expressing this enzyme at basal or activated level can be selected using nickel chloride (NiCl₂) or tetracycline, respectively. First, the strength of the promoter for riboswitch expression and the extent of the positive (tetracycline) and negative (NiCl₂) selection pressures were optimized. Various concentrations of tetracycline or NiCl₂ were supplemented to the culture broths of E. coli strains that contained the plasmids expressing different amounts of *tetA-sGFP* by the Anderson promoter series. The specific growth rates of the strains were measured (Figure S3). BBA_J23113 promoter was chosen for its low expression level, because the expression of riboswitch and riboswitch-encoded gene at higher level may affect physiology of the host cell. The promoter with the lowest expression level, BBA_J23112, was excluded because the negative selection was unable to discriminate this promoter from BBA_J23113. The optimal selection pressures were decided as 50 μg/mL tetracycline and 10 mM NiCl₂ for the positive and the negative selection, respectively.

For enrichment of the riboswitch library, an overnight culture of E. coli W3110 transformed with the riboswitch library plasmid was inoculated into 3 mL of M9 medium containing 34 μg/mL chloramphenicol (CM9) at an OD₆₅₀ of 0.05. After cultivation at 37 °C for 8 h with shaking (200 rpm), the culture broth was inoculated into 3 mL of fresh CM9 with an appropriate selection pressure at an OD₆₅₀ of 0.01. The culture broth for the Mid selection route was enriched in the following sequence: negative (0 mg/L naringenin), negative (10 mg/L naringenin), and positive (100 mg/L naringenin). In contrast, the riboswitches for the High selection route were enriched in the following sequence: negative (0 mg/L naringenin), negative (10 mg/L naringenin), negative (100 mg/L naringenin), and positive (200 mg/L naringenin). Either 10 mM NiCl₂ or 50 μg/mL tetracycline were supplemented to the medium for negative or positive selection, respectively, and each selection step was terminated when the OD₆₅₀ reached 0.5. The whole selection sequences were repeated twice for each route.

After all the enrichments, culture broth from each selection route was spread on a Luria broth agar plate containing 34 μg/mL chloramphenicol and incubated at 37 °C overnight to obtain single colonies. Ten colonies from each selection route were cultured in CM9 with or without 200 mg/L naringenin. Then, the specific fluorescence was calculated by normalizing the fluorescence intensity to the OD₆₅₀ of each culture broth. Three colonies with the highest specific fluorescence were chosen from each selection route for further characterization (Figure S4). The OD₆₅₀ was measured using the UV-1700 spectrophotometer, and the fluorescence was measured using a VICTOR³ 1420 multilabel counter (PerkinElmer, Waltham, MA). Fluorescence was detected using a 486 nm excitation filter and a 535 nm emission filter with 1 s measurement time. The Mann–Whitney rank sum tests for the comparison of the fold activations and the expression levels of the riboswitches were performed using SigmaPlot software (Systat Software, Inc., San Jose, CA).

**Fitting of the Dose–Response Curves and Evaluation of the EC₅₀s.** The E. coli W3110 cells that contain the selected riboswitches were cultured at 37 °C in a BioscreenC plate (Oy Growth Curves Ab, Helsinki, Finland) containing 200 μL of CM9 supplemented with naringenin of various concentrations (0, 10, 50, 100, 150, and 200 mg/L). Supplementation of naringenin to higher concentrations was impractical due to its
low solubility. All cultures were performed in biological triplicates. Fluorescence was measured using the VICTOR® and normalized to the OD$_{600}$ of each culture broth measured by the BioscreenC. Dose–response curves of the selected riboswitches were plotted using the fold activation calculated by normalizing the specific fluorescence to the specific fluorescence when no naringenin was added to the medium. The plots were fitted using the following equation: Fold activation = Minimum + (Maximum − Minimum)/(1 + $10^{(log(C_{50})−log([NBR])})$). The EC$_{50}$ values were estimated from the fitting results. Fitting of the dose–response curves and the estimation of the EC$_{50}$ values were performed using SigmaPlot software (Systat Software, Inc., San Jose, CA).

sGFP Expression Assay To Determine the Specificity of the Riboswitches. The specificity of the riboswitches was tested in E. coli BL21star(DE3). Single colonies of each strain were inoculated into 10 mL of LB with 25 μg/mL chloramphenicol in a 125 mL nonbaffled flask and grown overnight at 37 °C with shaking. After 14 h, the culture broth was inoculated at 2% (40 μL) into 2 mL of CM9. Naringenin (Sigma-Aldrich, St. Louis, MO), eriodictyol (Indolne Chemical Company, Inc., Hillsborough, NJ), flavone (Sigma-Aldrich, St. Louis, MO), luteolin (Sigma-Aldrich, St. Louis, MO), and apigenin (Indolne Chemical Company, Inc., Hillsborough, NJ) were added to a final concentration of 0.2 mM, and the strains were cultured for 5 h. All cultivation was performed in polypropylene 48-well plates (5 mL, VWR).

Flow cytometry was performed using the LSRII flow cytometer (BD Biosciences, San Jose, CA) with 488 nm excitation from a blue solid-state laser. Fluorescence was detected using a 505 nm long-pass and a 530/30 nm band-pass filter set for sGFP. Cells collected by centrifugation (2 min, 20 000 × g) were washed and diluted in the cold phosphate buffered saline (PBS) and kept on ice until further use. Gating was performed on forward and side scatter to avoid debris and clumped cells using the BD FACSDiVa 7.0 software. The specific fluorescence was defined as the geometric mean of the fluorescence from 100 000 cells, and the values were normalized to the specific fluorescence observed in the presence of naringenin. The heat map and hierarchical clustering were generated using a web-based tool (https://software.broadinstitute.org/morpheus/). The clustering utilized Pearson correlation with complete linkage method.

The specificity of Mid3 riboswitch was tested in E. coli BL21star(DE3)ΔsucCΔfumC/pFlavo$^{opt}$. This strain was able to produce naringenin or eriodictyol using p-coumaric acid or caffeic acid as substrates, respectively. We removed two T7 promoters and a lacI gene contained in the original Mid3 riboswitch plasmid, because IPTG (isopropyl β-D-1-thiogalactopyranoside) induction may affect the expression of the riboswitch. The plasmid was amplified with PCR using two sets of primers: NNRNS_F/R and pACYC_F/R. Then, the DNA fragments were digested by Bsal and ligated using the Quick ligase kit (New England Biolabs, Ipswich, MA) and transformed into E. coli Mach1-T1R cells. The modified pMid3 plasmid was transformed to either the producer strain or to the background strain without pFlavo$^{opt}$ plasmid. The culture was performed as described in the reference. Single colonies of each strain were inoculated to 3 mL of AMM medium supplemented with 34 μg/mL chloramphenicol (C-AMM). For the strain with pFlavo$^{opt}$, 80 μg/mL ampicillin was additionally supplemented (AC-AMM). The strains were cultivated at 37 °C for 14 h with shaking (200 rpm). Then, the culture broths were inoculated to 3 mL of fresh medium at an OD$_{600}$ of 0.08 and cultured at 37 °C for 4 h with shaking (200 rpm). The refreshed culture broths were inoculated again to 3 mL of fresh medium at an OD$_{600}$ of 0.08 and cultured at 37 °C for 4 h with shaking (200 rpm). The pathway genes were induced with 1 mM IPTG, and 0.6 mM of substrate (p-coumaric acid or caffeic acid) was added to medium. Fluorescence and OD$_{600}$ were measured after cultivation at 30 °C for 12 h with shaking (200 rpm). The fluorescence was normalized by the OD$_{600}$ to calculate specific fluorescence. All experiments were performed in biological triplicates.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.7b00128.

Overall flowchart of the in vitro and in vivo selections used in this study (Figure S1); Coupling of naringenin to epoxy-activated sepharose 6B (Figure S2); Optimization of the selection pressures (Figure S3); Fold activation of the enriched E. coli strains from the “Mid” and “High” selection routes (Figure S4); Dose–response curves of the naringenin riboswitches (Figure S5); Secondary structures of Mid2 and High2 riboswitches (Figure S6); Sequences of the naringenin riboswitches (Table S1); Oligonucleotides used in this study (Table S2); Strains and plasmids used in this study (Table S3) (PDF).

AUTHOR INFORMATION

Corresponding Author

E-mail: gyjung@postech.ac.kr.

Orcid

Mattheos A. G. Koffas: 0000-0002-1405-0565

Gyoo Yeol Jung: 0000-0002-9742-3207

Author Contributions

Sungho Jang and Sungyeon Jang contributed equally to this work.

Notes

The authors declare no competing financial interest.

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