

Toehold Switch-Based Genetic Modulator for Tuning of Riboswitch Sensor

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Research

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Abstract

Background

Synthetic biological circuits are widely utilized to control microbial cell functions. Natural and synthetic riboswitches are attractive classes of sensor modules for use in synthetic biological applications. However, tuning the dose-response parameters of riboswitch circuits is challenging because considerable understanding of riboswitch mechanism and screening of mutant libraries are generally required. Therefore, novel molecular parts and strategies for controlling the dose-response parameters of riboswitch circuits are needed.

Results

Here, we developed a toehold switch-based genetic modulator that combines a previously reported hybrid input construct, which consists of riboswitch and transcriptional repressor, and *de-novo*-designed riboregulators named as toehold switches. First, the introduction of a pair of toehold switch and trigger as a downstream signal-processing module resulted in a functional riboswitch circuit. Next, several optimization strategies that focused on the stoichiometric ratio of RNA components greatly improved the fold-change. Finally, further characterizations confirmed low leakiness and high orthogonality for multiple toehold switches, indicating its applicability in riboswitch circuits in a seamless manner.

Conclusions

The toehold switch-based genetic modulator improved the dynamic range and dramatically shifted the operational range compared to the previous sensors only with hybrid input construct. The programmable RNA-RNA interactions amenable to *in silico* design and optimization can facilitate further development of RNA-based genetic modulators for flexible tuning of riboswitch circuitry and synthetic biosensors.

Introduction

Synthetic biology is an emerging engineering discipline that aims to design and build biological parts, devices, and systems based on the understanding of biological systems [1]. One important synthetic biology research direction is to embed synthetic biological circuits in microbial cells to control their responses to environmental inputs, mainly by designing novel genetic circuits [2]. Simple genetic parts are assembled to construct complex genetic circuits with useful functions, and numerous applications utilizing genetic circuits have been reported such as monitoring of small molecules, control of metabolic pathways, directed evolution of enzymes, and logic computation [3–6].

A riboswitch is an RNA-based regulator composed of an aptamer domain capable of binding a ligand and an expression platform that undergoes structural changes in response to the ligand binding to the aptamer. The riboswitch is a *cis*-acting regulator since it controls the expression of the gene in the same mRNA in which the riboswitch is encoded [7, 8]. Riboswitches have been utilized as an input part to

construct synthetic genetic circuits for a variety of applications. Particularly, natural and artificial riboswitches have been widely utilized to monitor intracellular metabolite concentrations and in turn regulate the expression of functional genes. For instance, riboswitches enabled examination of the metabolism and transport of certain metabolites [9, 10], high-throughput screening and selection of metabolite-producing microbial strains [11, 12], directed evolution of enzymes [13], and evolution of a useful phenotype [14]. The performance of this regulator can be characterized by dose-response parameters such as the dynamic range and operational range. The dynamic range refers to the range of output signal level from its minimum to maximum, and the ratio of the minimum and maximum output signals is called fold-change. The operational range refers to the range of ligand concentration where the change in ligand concentration causes the difference in the output signal level. Ideally, dose-response parameters of riboswitches should be tunable to maximize their effectiveness. First, the fold-change of an output signal in response to an input signal should be as high as possible. Tight and strong gene expression regulation is highly desirable to maximize the regulatory outcome while minimizing leaky expression that can contribute to the gene expression noise and unnecessary consumption of cellular resources. Further, the operational range must encompass the expected ligand concentration range, which is specific to each application [3, 5]. In particular, to improve metabolite-producing microbial strains, biosensors that operate at high concentrations of ligands need to be engineered because the operational range of biosensors is often significantly lower than the chemical productivity of the optimized strains [15–17].

Several approaches have been reported for tuning the dose-response of riboswitches. Previous studies showed that modifying binding kinetics and affinity by directed mutagenesis of aptamer domains of riboswitches shifted the dose-response curves [18–20]. Another approach focused on modifying an expression platform, which was also effective for tuning of riboswitches [21, 22]. However, these engineering approaches typically require a detailed understanding of the structure, biochemistry, and evolution of a riboswitch. Tuning the dose-response of a riboswitch generally involves screening of mutant libraries, even for those that have been molecularly characterized, making the tuning process laborious and time-consuming. An alternative way to adjust the dose-response parameters while maintaining the biosensor sequence is to insert new genetic regulation steps. The dose-response parameters of the resulting riboswitch circuit may be limited by the properties of downstream signal processing module. Still, it is plausible that the dose-response parameters of riboswitch circuit can be tuned beyond the limit of natural riboswitches by using multi-level binding events in the downstream signal-processing module. For example, we previously reported a hybrid input riboswitch circuit that combined a natural riboswitch and transcriptional repressors [23]. The hybrid input inverted the output signal from the riboswitch and amplified its fold-change from 7.5- to 32.1-fold without extensive characterization or domain modifications of the riboswitch. However, the fold-change was still limited compared to optimized transcription factor-based circuits that can show dynamic range up to several hundred folds. Further, only a modest change in the operational range was observed for this hybrid input strategy, in which transcriptional repressor alone was not sufficient to tune the operational range.

Therefore, novel molecular parts and design strategies are required to adjust the operational range of riboswitch circuits for diverse and demanding applications.

Progress in RNA synthetic biology has provided a multitude of readily usable novel parts that may be integrated with existing synthetic circuit designs. We focused on a new type of RNA-based regulator known as a toehold switch, which provides a library of *de-novo*-designed regulatory parts with large dynamic ranges and high orthogonality [24]. The ribosome binding site of a toehold switch is exposed upon specific binding to a cognate trigger RNA, allowing for precise control of gene expression at the post-transcription level [24, 25]. We hypothesized that the toehold switch can be used to adjust the dose-response properties of riboswitch circuits by inserting another signal propagation stage. The large dynamic range of toehold switches may further amplify the output signal from the riboswitch circuits. Further, programmable toehold switches and trigger pairs with different ranges of binding affinities may provide another tuning knob for the operational range upon insertion in the riboswitch circuitry.

In this study, we showed that the toehold switch can be utilized to modulate the dose-response of a riboswitch-based sensor. Previously reported hybrid input parts for coenzyme B₁₂ were combined with toehold switch-trigger pairs. The dose-response curves of the resulting circuits showed a substantial improvement in the fold-change and a shift in operational range. These results demonstrate that toehold switches can provide programmable and modular plug-and-play genetic parts for the response tuning of riboswitch circuitry.

Methods

Strains, plasmids, and oligonucleotides

The strains and plasmids used in this study are listed in Table S1 and the oligonucleotides used are listed in Table S2. Sequencing of the constructed plasmids and synthesis of oligonucleotides were performed by Cosmogenetech (Seoul, Korea).

Bacterial strains and genetic circuit construction

Because the plasmid that regulates expression of the trigger by coenzyme B₁₂ should not contain the T7 promoter, the two T7 promoter sites in pACYCDuet-1 were deleted. The plasmid was PCR-amplified using Q5 High-Fidelity DNA Polymerase (New England Biolabs (NEB), Ipswich, MA, USA) with 5'-end phosphorylated primers followed by blunt-end ligation with Quick Ligase (NEB). T7-1-remove-F, R and T7-2-remove-F and R were used, respectively. Phosphorylation at the 5' end was performed using T4 Polynucleotide Kinase (Takara, Shiga, Japan).

The coenzyme B₁₂ sensing module and trigger moiety of pET-trN1 were inserted into pACYC-dT7 with T7 promoter deletion [25]. The plasmid pB12ribo-J23100-PhIF was used as a template for amplification of the insert using pACYC-gibson-In-F/trN1-R for trN1, where the trigger sequence was included as overhangs in the primers. The vector was amplified using pACYC-dT7 as a template using pACYC-gibson-

Ve-R/trN1-F as primers. These amplified fragments were ligated by the Gibson Assembly method using NEBuilder® HiFi DNA Assembly Master Mix (NEB). The resulting coenzyme B₁₂ sensing module, pACYC-B12ribo-PhIF-trN1, was co-transformed with pCOLA-swN1-GFP to construct the trN1-swN1 strain.

Plasmids that additionally express the trigger RNAs (pACYC-B12ribo-PhIF-trN1_J23106, J23101, and J23100-trN1) were constructed by the Gibson Assembly method. pACYC-B12ribo-PhIF-trN1 was PCR-amplified using the primers ConstP-Vec-F/R to prepare vector DNA fragment. Insert DNA fragments that have a constitutive promoter and trigger sequence were PCR-amplified using pACYC-B12ribo-PhIF-trN1 as a template, ConstP-In-F as a forward primer, and ConstP-106, 101, and 100-In-R as reverse primers. The resulting plasmids, pACYC-B12ribo-PhIF-trN1_J23106, J23101, and J23100-trN1, were co-transformed with pCOLA-swN1-GFP to construct the trN1-swN1_J23106, J23101, and J23100 strains.

The pACYC-B12riboPhIF-trN1 was used as a template for PCR to change the trigger sequence in the coenzyme B₁₂ sensing module. DNA fragments with replaced sequences were amplified using the primers Tri-over-trN3-F/R, tr1N2-F/R, trN2-F/R, and trN6-F/R, respectively, and plasmids were constructed by blunt-end ligation. The resulting plasmids, pACYC-B12ribo-PhIF-trN3, tr1N2, trN2, trN6, were co-transformed with pCOLA-swN3, sw1N2, swN2, and swN6-GFP, respectively, to construct the trN3-swN3, tr1N2-sw1N2, trN2-swN2, and trN6-swN6 strains. Additionally, the plasmid pACYC-B12ribo-PhIF-deltr was constructed by blunt-end ligation of the PCR product which was amplified by using pACYC-B12ribo-PhIF-trN1 as a template and del-tr-F/R as primers. All genetic circuit systems were tested using *Escherichia coli* BL21 Star (DE3) except for those shown in Figure S3 for which *E. coli* BL21 (DE3) was used.

Fluorescence measurement with coenzyme B₁₂

All cultivation experiments were performed using M9 medium containing glucose (4 g/L glucose, 6.78 g/L disodium phosphate (anhydrous), 3 g/L monopotassium phosphate, 0.5 g/L sodium chloride, 1 g/L ammonium chloride, 2 mM magnesium sulfate, 0.1 mM calcium chloride) and appropriate antibiotics (27 mg/L chloramphenicol and 40 mg/L kanamycin). The strains were incubated at 37 °C with shaking at 200 rpm. Single colonies were inoculated into M9 medium, cultured for 24 h, and diluted to a final OD₆₀₀ of 0.05 in fresh M9 medium. Seed cultures in mid-log phase were adjusted to an OD₆₀₀ of 0.05 in fresh M9 medium and incubated for 12 h. Coenzyme B₁₂ (Sigma-Aldrich, St. Louis, MO, USA) was added at different concentrations (0, 0.1, 0.3, 1, 3, 10, and 30 μM). Toehold switches connected to GFP reporters were expressed in BL21 Star (DE3) cells, an RNase-deficient strain, or in BL21 (DE3) cells, a non-RNase-deficient strain, with the T7 RNA polymerase induced by adding IPTG at 10 μM unless stated otherwise.

The fluorescence and OD₆₀₀ of the cells were measured with a VICTOR³ 1420 Multilabel Counter (PerkinElmer, Waltham, MA, USA). First, cell pellets were washed with phosphate-buffered saline (PBS) and resuspended in PBS. Fluorescence was then measured using an 485-nm excitation filter and 535-nm emission filter with a 0.1-s measurement time, and the OD₆₀₀ was determined using a 600-nm filter with a

0.1-s measurement time. The OD₆₀₀ and fluorescence values were corrected by subtracting the values measured for PBS. Autofluorescence of the cells was not subtracted from the fluorescence value.

Fitting of dose-response curves and calculation of EC₅₀

SigmaPlot software (Systat Software, Inc., San Jose, CA, USA) was used to fit the dose-response curve. Data were fitted using a nonlinear regression – dynamic fitting program, and an equation of ligand binding and sigmoidal dose-response was selected. The EC₅₀ value was calculated using the fitting results, and the following logistic equation was used: Fluorescence = Min. + (Max. - Min.)/(1 + 10^{(log(EC50)-log(coenzyme B12))×(Hill coefficient)}).

Results And Discussion

Construction of toehold switch-based genetic modulator

To construct an RNA-based genetic modulator, we evaluated toehold switches as modular plug-and-play genetic parts in the riboswitch circuitry. One pair of toehold switch and trigger RNA, AND-computing toehold switch ACTS_Typell_N1, was selected based on its high fold-change [25] and inserted into previously characterized coenzyme B₁₂-responsive hybrid input riboswitch circuit (Fig. 1) [23]. The hybrid input riboswitch circuit combined an off-type coenzyme B₁₂ riboswitch from the 5'-untranslated region of *cbiA* from *Salmonella typhimurium* [26] and transcriptional repressors [27] to invert the output signal from the riboswitch and amplify the response. The input coenzyme B₁₂ down-regulates the expression of the transcriptional repressor under control of the riboswitch, which in turn activates the expression of the final reporter gene under control of the transcriptional repressor. Previously, several constitutive promoter and transcriptional repressor combinations were tested to modulate the dose-response of riboswitch circuits, and a variant named as P100 composed of the BBa_J23100 promoter and PhIF transcriptional repressor showed the highest fold-change of 32.1 [23].

We chose the P100 as the target riboswitch regulatory circuit to be modified based on its previously demonstrated performance. A direct connection formed by the PhIF transcriptional repressor and its cognate promoter driving the green fluorescent protein (GFP) reporter was converted to an indirect connection by introducing toehold switch-trigger pairs. Under this new scheme, the PhIF-cognate promoter drives the expression of trigger RNA (trN1, moiety of trigger RNA sequence in ACTS_Typell_N1) rather than reporter genes. Additionally, the reporter gene was introduced downstream of a toehold switch that specifically recognizes its cognate trigger RNA (swN1, moiety of switch RNA sequence in ACTS_Typell_N1). The engineered circuit (trN1-swN1) was transformed into the *E. coli* BL21 Star (DE3) strain, which is RNase-deficient, and the performance of this new riboswitch circuit was evaluated (Fig. 2a, b). The GFP reporter expression was increased by 18.1-fold, in the presence of 30 μM coenzyme B₁₂ and 100 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Fig. 2b). The toehold-switch-based riboswitch circuit showed expected activation of GFP expression in the presence of coenzyme B₁₂, but the

fold-change was less than the reported values of P100 circuit. Several factors that can contribute to the limited dynamic range and various optimization strategies will be investigated in the next section.

Before tuning the dynamic range, we assessed the operational range of the toehold-switch-based riboswitch circuit. The operational range of an intracellular biosensor is critical for its successful application in metabolic engineering, such as in high-throughput screening of metabolite producers [5, 28]. For synthetic riboswitch circuits, the operational range and half-maximal effective concentration (EC_{50}) are typically determined by the binding affinities between the genetic circuit components that are not easy to be adjusted [29]. Alternatively, the dose-response can be adjusted by allowing the final output signal to be expressed through multi-step binding events. For example, the dose-response parameters were effectively controlled when the final output signals were expressed through two or three steps of the binding events in transcriptional cascades [30]. In the case of P100 riboswitch circuit, the dose-response parameters could be adjusted by connecting a transcriptional repressor to the natural coenzyme B_{12} riboswitch in series. Despite efforts to tune the operational ranges by adjusting the promoter strengths of the riboswitch and transcriptional repressors used, a limited effect on the EC_{50} of the circuit was observed [23].

We hypothesized that the operational range of a synthetic genetic circuit can be further adjusted by using a genetic modulator strategy that utilizes a new type of molecular interaction for signal propagation. In the toehold-switch-based modulators developed in this study, a new type of RNA-RNA molecular interaction was introduced on top of the protein repressor used in previous hybrid input riboswitch circuits. Therefore, we measured the output signal from the engineered circuits over a wide range of coenzyme B_{12} concentration to evaluate the operational range (Fig. 2c). The toehold-switch-based modulator circuit activated gene expression at coenzyme B_{12} concentrations ranging from 0.3 to 10 μ M. Compared to the operational range of the previous P100 circuit (0.3–10 nM), introduction of the toehold-switch-based modulator dramatically shifted the dose-response curve by three orders of magnitude. The EC_{50} value of trN1-swN1 was 3,455 nM (Figure S1), which was a substantial increase compared to the EC_{50} of P100 (1.52 nM).

Toehold switch-based modulator tuning by adjusting stoichiometric ratio between switch RNA and trigger RNA

The RNA-RNA interaction of switch and trigger molecules is the key feature in the toehold switch-based modulator, and therefore we asked whether adjusting the stoichiometric ratio between the trigger RNA and switch RNA could impact the dose-response curves. First, the effect of adjusting switch RNA expression on dose-response was explored: overexpression of switch RNA would increase the basal expression of the GFP reporter, while very low expression of switch RNA would lower the overall performance of genetic circuitry. Accordingly, a range of switch RNA levels was explored by different IPTG concentrations (Fig. 3a). Both the minimum and maximum signals tended to decrease with decreased switch RNA concentrations (Table S3). The fold-change was greatly improved, reaching over 200-fold under 10 μ M IPTG condition, mainly due to a substantial decrease in leaky GFP expression in the absence

of coenzyme B₁₂ (Fig. 3b). As the switch RNA were induced more strongly, the EC₅₀ values decreased (Figure S2), likely because a larger amount of switch RNAs can detect trigger RNA more sensitively. Second, the impact of expression level of trigger RNA on the performance was evaluated: the expression of trigger RNA under the control of coenzyme B₁₂ module may not be sufficient to maximize the dynamic range. Accordingly, an additional copy of trigger RNA was expressed under the control of constitutive promoters to adjust the basal expression of trigger RNA (Fig. 3c). A trend was observed where the additional copy of trigger RNA under a strong constitutive promoter led to higher maximum signal (Table S3). However, the basal expression of GFP reporter increased as well, resulting in a similar level of fold-change (Fig. 3d). Overall, the dose-response was tunable by stoichiometric changes of switch and trigger RNAs while maintaining the same switch-trigger pairs.

Leakiness, orthogonality and modularity of toehold switch-based modulators

We introduced another toehold switch pair (trN3-swN3) to the P100, and investigated the role and specificity of the trigger RNAs used in the coenzyme B₁₂ riboswitch circuits. When trigger RNAs downstream of the PhIF-cognate promoter were deleted, the circuits showed low basal expression of the reporter outputs upon introduction of coenzyme B₁₂ inputs (Fig. 4a, swN1 and swN3). Additionally, the circuits with non-cognate trigger-toehold switch pairs (trN3-swN1 and trN1-swN3) were not responsive to coenzyme B₁₂, which shows that the leakiness of these circuits are very low. Cognate trigger and switch pairs produced robust GFP expression, resulting in ON/OFF levels over 300- and 100-fold, respectively (trN1-swN1 and trN3-swN3). Together, these results indicate that the operation of the engineered coenzyme B₁₂ riboswitch circuit requires specific binding of the trigger RNA and its cognate toehold switch, which is consistent with the strong orthogonality and high fold-change of toehold switches demonstrated in other studies [24, 25].

Next, we further evaluated the modularity of the toehold switch modulator, and the impact of toehold switch elements on the dose-response parameters. Three additional toehold switch pairs were introduced to the P100 circuit in the same manner, and the dose-responses were evaluated. These new constructs showed distinct dynamic ranges depending on the toehold switch pairs introduced (Fig. 4b). The fold-changes ranged from 90 to 346 for different devices, indicating that the dynamic range of toehold switch modulator is comparable to transcription factor-based circuits (Fig. 4c). This increase in the fold-change is likely related to the high fold-change of the toehold switch and trigger pairs used in the circuits. We observed that the operational range remained similar, with similar EC₅₀ values, which may be limited by the intrinsic characteristic of the current toehold switch design (EC₅₀ values not shown). In addition, to test compatibility of these sensors in various strains, we confirmed that the circuits also work in a non-RNase-deficient strain (Figure S3). Because an intact RNase E gene is present in strain BL21 (DE3), both the minimum and maximum output levels were lower than those observed in the RNase-deficient strain BL21 Star (DE3). Despite apparent decreases in signals for strain BL21 (DE3), the fold-changes of the circuits were comparable to strain BL21 Star (DE3).

We used a toehold-switch-based modulator to tune the dose-response curves in a modular fashion without the requirement for previous knowledge of the riboswitch within the synthetic circuit. In particular, the fold-change was substantially improved over previous implementation of hybrid riboswitch circuit, and the dynamic range can be further adjusted by multiple tuning strategies. At the same time, the EC_{50} values were shifted to high values as compared to other engineering approaches. While we provide some examples of high-performance toehold switches and trigger RNA pairs in this study, an extended library of more than 100 toehold switches has been reported with more than 20 toehold switch-trigger pairs with large dynamic range and high level of orthogonality [24, 25, 31, 32]. Therefore, the strategy of using the toehold-switch-based genetic modulator is highly modular and scalable for future efforts to tune the dose-response of genetic circuits to construct more complex genetic programs.

Conclusion

We demonstrated that toehold switch-based genetic modulator can tune the dynamic range of riboswitch circuits and dramatically shifted the operational range. The successful incorporation of several pairs of toehold switches, multiple tuning strategies including stoichiometric adjustments provide evidence for more general applicability of the proposed modulator strategy. In the future, through combined efforts including mathematical and thermodynamic modeling, the toehold switches may be able to provide modular plug-and-play genetic parts for tuning of riboswitch circuitry, expanding the range of applications of synthetic biosensors.

Declarations

Competing interests

The authors declare no competing interests.

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Author contributions

Y. Hwang, S.G. Kim, and S. Jang contributed equally to this work. Y. Hwang, S.G. Kim, S. Jang, J. Kim, and G.Y. Jung designed the study, analyzed the data, and wrote the manuscript.

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Figures

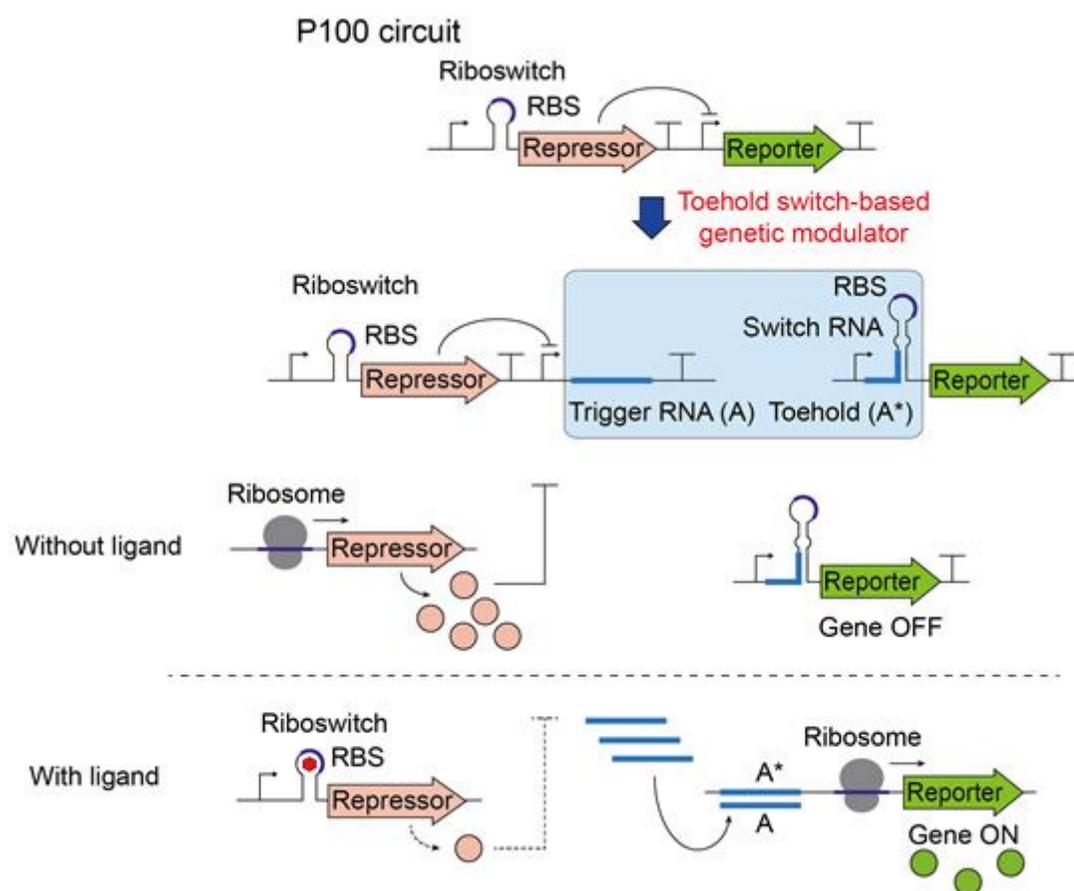


Figure 1

Overall scheme of the toehold switch-based genetic modulator. A previously characterized hybrid input riboswitch circuit (P100) combined an off-type coenzyme B12 riboswitch with transcriptional repressors to invert the output signal [23]. In the toehold switch-based genetic modulator, the riboswitch controlled the expression of transcriptional repressors; however, the transcriptional repressors regulated the expression of trigger RNAs, rather than reporter proteins. The interaction between the toehold switch and trigger RNA pairs activates the expression of the reporter gene. In the absence of ligand, the transcriptional repressor is expressed and represses the transcription of trigger RNA, causing the toehold switch RNA to maintain a translation-repressing hairpin structure, occluding the ribosomal binding site (RBS) of the reporter gene. In the presence of ligand, expression of the transcriptional repressor is inhibited, resulting in strong transcription of trigger RNAs; the binding of trigger RNA to the cognate toehold switch RNA exposes the RBS to activate translation of the reporter gene.

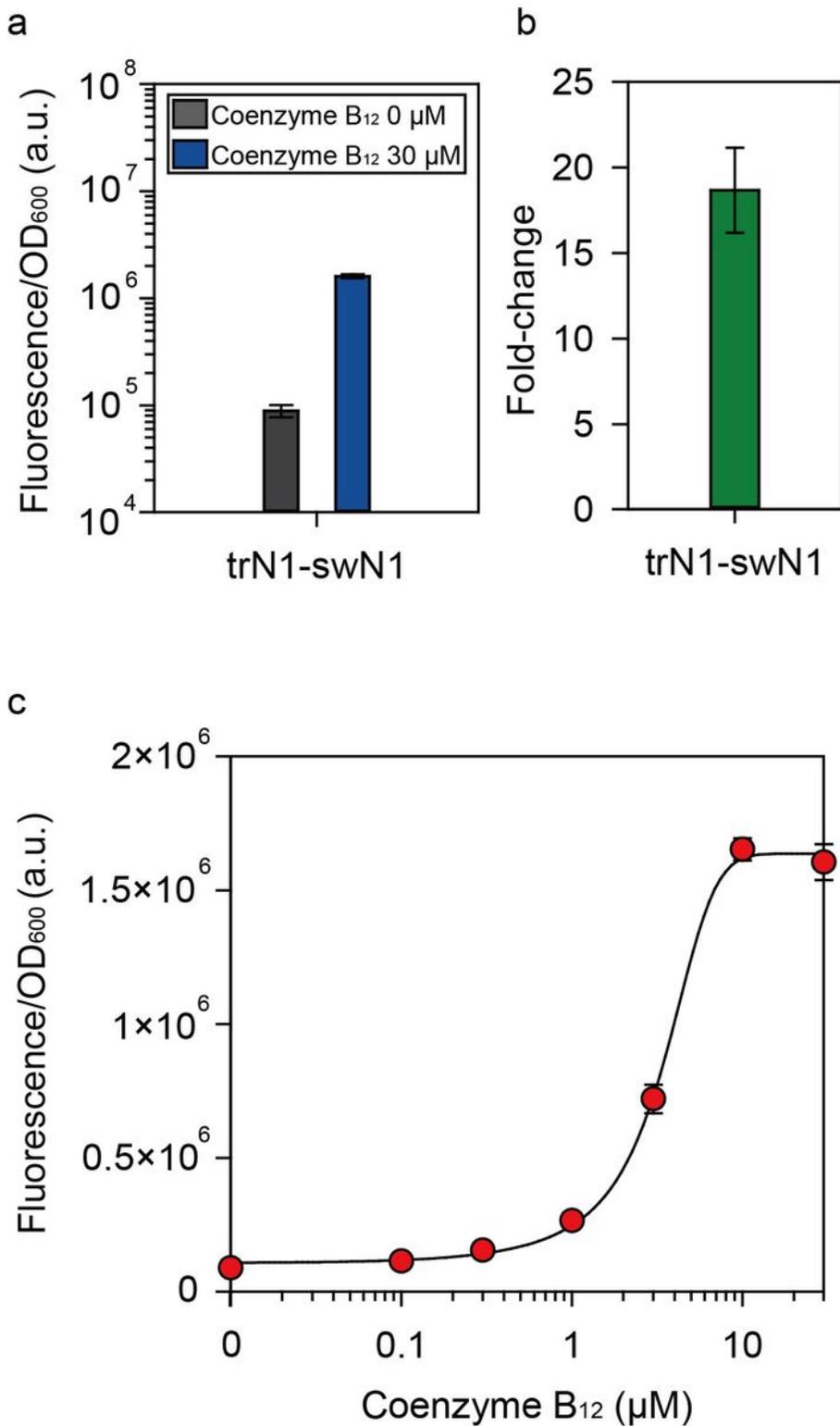


Figure 2

Properties of riboswitch circuits engineered with toehold switch-based genetic modulators. (a) Fluorescence measurements for engineered circuits with cognate trigger and toehold switch pair (trN1-swN1) with and without coenzyme B₁₂ (30 μM was added). (b) Fold-change of GFP reporters for trN1-swN1 of (a). (c) Dose-response curve of trN1-swN1. Coenzyme B₁₂ was added at different

concentrations (0, 0.1, 0.3, 1, 3, 10, 30 μM), and IPTG was added at 100 μM . Error bars indicate standard deviations from biological triplicate measurements.

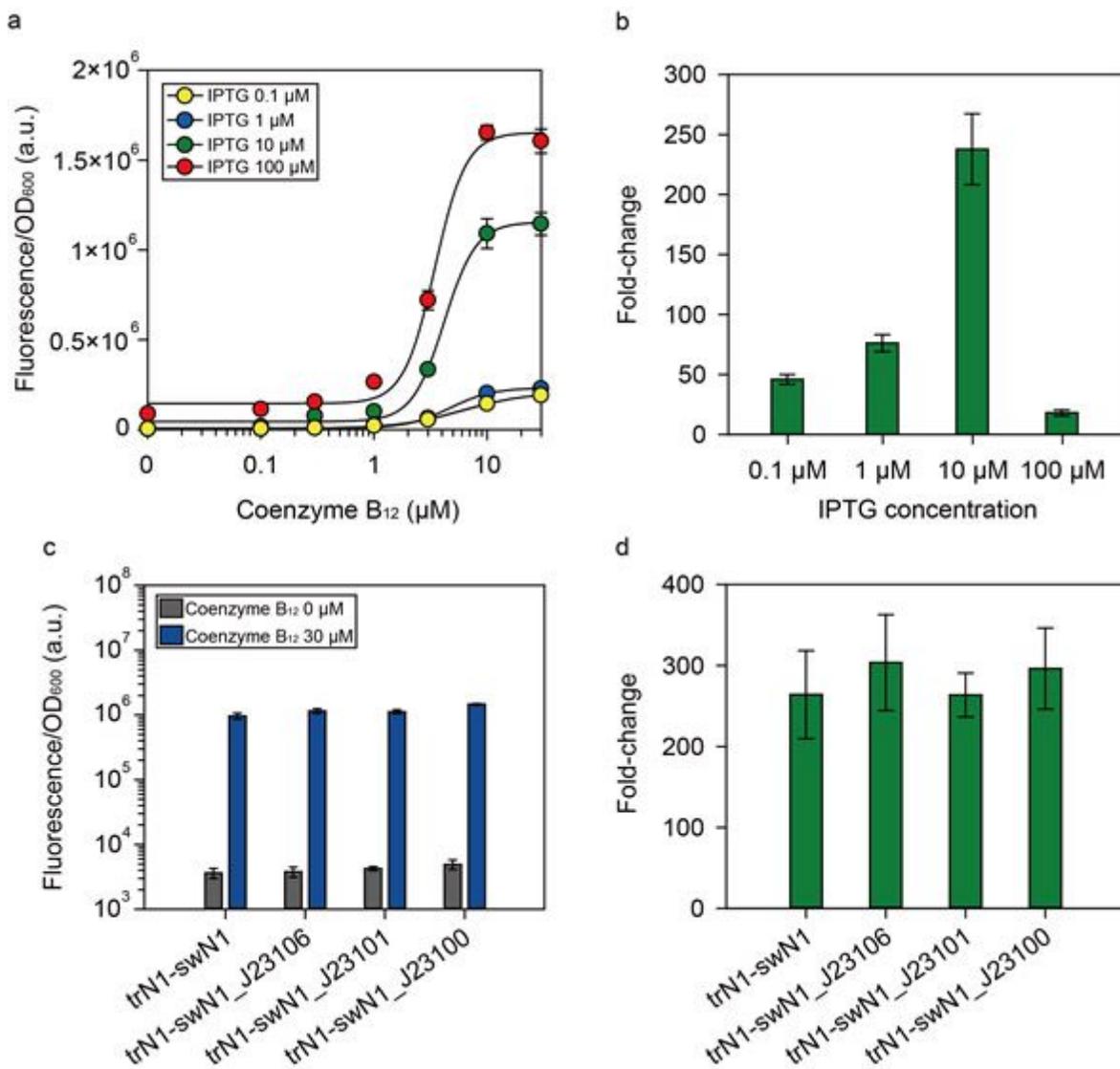


Figure 3

GFP fluorescence and fold-change results by adjusting the amount of switch RNA or trigger RNA. (a) Dose-response curves of trN1-swN1 with different concentrations of IPTG (0.1, 1, 10, 100 μM). Different concentrations of coenzyme B12 (0, 0.1, 0.3, 1, 3, 10, 30 μM) were used at each experiment. The R² values of the fits were calculated as 0.9990 (trN1-swN1 with 0.1 μM IPTG), 0.9936 (trN1-swN1 with 1 μM IPTG), 0.9948 (trN1-swN1 with 10 μM IPTG), 0.9959 (trN1-swN1 with 100 μM IPTG) respectively. (b) Fold-change of GFP reporters for the strains in (a). (c) Fluorescence measurements for trN1-swN1 and modified trN1-swN1 strains that express additional trigger RNAs under several constitutive promoters with and without coenzyme B12 (30 μM was added). Three constitutive promoters (The Anderson promoter collection: BBa_J23106 (1185 a.u.), BBa_J23101 (1791 a.u.), and BBa_J23100 (2547 a.u.)) were used. (d) Fold-change of GFP reporters for the strains in (c). Error bars indicate standard deviations from biological triplicate measurements.

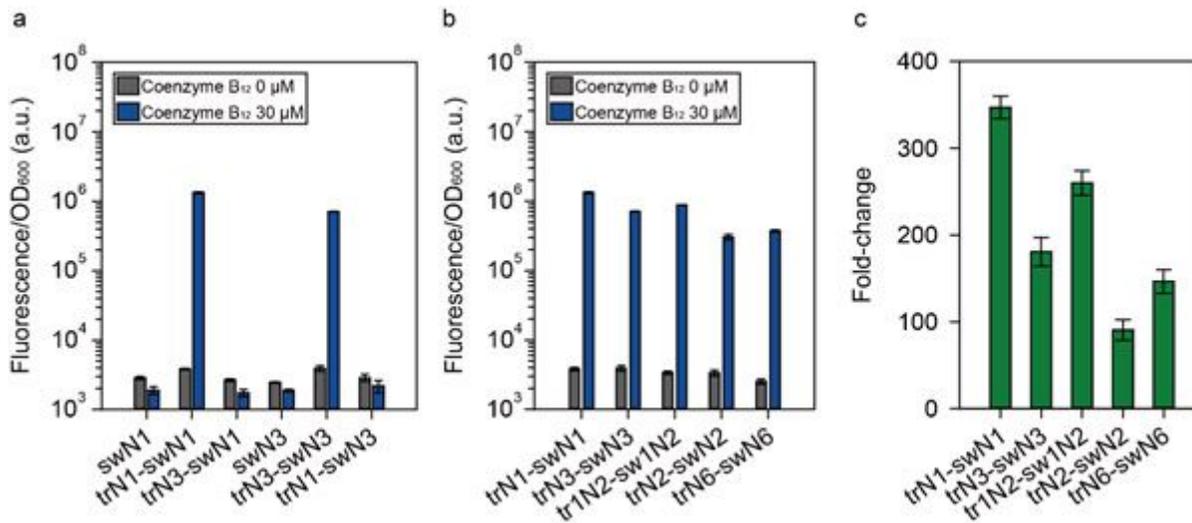


Figure 4

Characterizations of riboswitch circuits engineered with toehold switch-based genetic modulators. (a) Fluorescence measurements for combinations of toehold-trigger pairs and switch-only circuits with and without coenzyme B₁₂ (30 μM was added). (b) Fluorescence measurements of various pairs of toehold switch-based genetic circuits with and without coenzyme B₁₂ (30 μM was added). Three additional AND-computing toehold switches (ACTS_TypeI_N2, ACTS_TypeII_N2, and ACTS_TypeII_N6) were used [25]. (c) Fold-change of GFP reporters for the strains used in (b). Error bars indicate standard deviations from biological triplicate measurements.

Supplementary Files

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- [200417CoB12ToeholdSIMCF.docx](#)