Control of Gene Expression With Quercetin-Responsive Modular Circuits

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Control of gene expression is crucial for several biotechnological applications, especially for implementing predictable and controllable genetic circuits. Such circuits are often implemented with a transcriptional regulator activated by a specific signal. These regulators should work independently of the host machinery, with low gratuitous induction or crosstalk with host components. Moreover, the signal should also be orthogonal, recognized only by the regulator with minimal interference with the host operation. In this context, transcriptional regulators activated by plant metabolites as flavonoids emerge as candidates to control gene expression in bacteria. However, engineering novel circuits requires the characterization of the genetic parts (e.g., genes, promoters, ribosome binding sites, and terminators) in the host of interest. Therefore, we decomposed the QdoR regulatory system of B. subtilis, responsive to the flavonoid quercetin, and reassembled its parts into genetic circuits programmed to have different levels of gene expression and noise dependent on the concentration of quercetin. We showed that only one of the promoters regulated by QdoR worked well in E. coli, enabling the construction of other circuits induced by quercetin. The QdoR expression was modulated with constitutive promoters of different transcriptional strengths, leading to low expression levels when QdoR was highly expressed and vice versa. E. coli strains expressing high and low levels of QdoR were mixed and induced with the same quercetin concentration, resulting in two stable populations expressing different levels of their gene reporters. Besides, we demonstrated that the level of QdoR repression generated different noise levels in gene expression dependent on the concentration of quercetin. The circuits presented here can be exploited in applications requiring adjustment of gene expression and noise using a highly available and natural inducer as quercetin.

Keywords: genetic circuit, flavonoid, E. coli, quercetin, QdoR
INTRODUCTION

Cells naturally sense and react to extracellular signals. This environmental computation is carried out by transcriptional regulators that increase or decrease gene expression upon the appearance of a new molecule in the extracellular medium (or a variation of its concentration). Synthetic biologists can arrange these natural transcriptional regulators with synthetic genetic elements in genetic circuits to explore new functionalities. Recently, several genetic circuits have been assembled and characterized, enabling cells to respond to non-cognate signals (Wang and Buck, 2012; Ma et al., 2016; Xia et al., 2019).

Some soil bacteria naturally recognize metabolites produced and emitted by plants in their root exudates. For instance, Bacillus subtilis has the TetR-type negative regulator QdoR that is induced by flavonoids such as quercetin and fisetin (Hirooka et al., 2007). Flavonoids are versatile plant secondary metabolites that defend plants from invaders and signal beneficial soil microorganisms. One of the most abundant flavonoids is quercetin, which is produced in root exudates of Zea mays (maize) (Kidd et al., 2001), Arabidopsis thaliana (Narasimhan et al., 2003), and Alnus glutinosa (Hughes et al., 1999). Quercetin inhibits the supercoiling activity of DNA gyrase B and induces DNA cleavage in bacteria, resulting in growth inhibition (Plaper et al., 2003). B. subtilis avoids the harmful effects of quercetin by expressing the quercetin 2,3-dioxygenase QdoI, which inactivates quercetin by converting it to 2-protopcatechuyl-phloroglucinol carboxylic acid and carbon monoxide (Hirooka et al., 2007). QdoR represses the QdoI expression, binding to specific operators upstream of qdoI (Hirooka et al., 2007). QdoR also interacts with an operator upstream of qdoR, repressing its own expression (Hirooka et al., 2007; Hirooka and Fujita, 2011). Quercetin inhibits the binding of QdoR to DNA; thus, the transcription of qdoI and qdoR is induced (Hirooka et al., 2007).

The QdoR regulatory system was applied to construct biosensors to detect the intracellular concentration of quercetin in E. coli (Siedler et al., 2014) and monitor the quercetin content in soil (Del Valle et al., 2020). However, there were only a few modifications in the genetic elements of the native system, which did not allow modulating the inducer-response curves. Genetic circuits to sense naringenin, which belongs to another class of avonoids, were refactored with synthetic promoters to modulate gene expression mediated by quercetin. The circuits built in this work can potentially be applied to control expression in complex environments such as soil and the rhizosphere surrounding the roots of plants.

MATERIALS AND METHODS

E. coli Strains and Growth Conditions

E. coli TOP10 (Invitrogen, United States) was used for cloning purposes, while E. coli MG1655 (Blattner et al., 1997) was used as a chassis for testing the genetic circuits. The bacteria were grown in lysogeny broth (LB) (Sambrook and Russel, 2000) at 37°C, with shaking at 120 rpm (New Brunswick C25 Shaker), unless otherwise stated. The antibiotics were used in the following concentrations: ampicillin (250 μg ml⁻¹) and chloramphenicol (25 μg ml⁻¹).

Plasmids Construction

The plasmids used in this study were constructed using the BioBrick assembly method (Shetty et al., 2008). The genetic parts were obtained from the Registry of Standard Biological Parts or designed to contain the prefix and suffix of BioBricks Standard Assembly (RFC 10). The transcriptional factor qdoR, with codons optimized for E. coli expression by JCat software (Grote et al., 2005), was synthesized on-demand (Genscript, United States) with the incorporation of the prefix and suffix of BioBricks Standard Assembly. The promoters of qdoI (P_qdoI) and qdoR (P_qdoR) were obtained by cloning of annealed oligos, with the incorporation of the prefix and suffix of BioBricks Standard Assembly (Table 1) into the EcoRI and PstI sites of pSB1C3. P_qdoR corresponds to the sequence between the nucleotides 4,107,952 and 4,107,995, and P_qdoI between the nucleotides 4,107,278 and 4,107,327 in the genome of B. subtilis 168 (Kunst et al., 1997). Seeking to make P_qdoI compatible with the BioBricks RFC10 assembly, an XbaI site in the wild-type sequence was mutated. Hence, P_qdoI was synthesized with the following substitutions: 41 G > C and 42 A > T. Both substitutions lay between the -35 and -10 sites and outside the QdoR operators (Supplementary Table 1). From the Registry of Standard Biological Parts, we used synthetic constitutive promoters (BBa_J23114, BBa_J23115, BBa_J23116, BBa_J23105, and BBa_J23110), an RBS (BBa_B0034), a transcription terminator (BBa_B0015), and composite parts (BBa_I13504 and BBa_K1357010) formed by an RBS, gfp or rfp genes, and a transcription terminator. All the plasmids (Table 1) were constructed using pSB1C3 or pSB1A2 as backbone vectors (Registry of Standard Biological Parts), containing a pUC19-derived high copy replication origin and a chloramphenicol or ampicillin resistance marker. The DNA sequences of the genetic parts used in this work are provided in the Supplementary Material.

Cell Fluorescence Measurements

E. coli MG1655 cells transformed with a plasmid from Table 1 were inoculated and grown overnight at 37°C and 120 rpm. These cultures were diluted (1:100) in 200 μL of fresh LB medium and incubated in 96-well plates (Greiner Bio-One, 96 Flat Clear Bottom Black Polystyrene) to an optical density at 600 nm (OD₆₀₀) of approximately 0.7. Then, different concentrations of quercetin dissolved in dimethylsulfoxide (DMSO) were added. Fluorescence was followed during incubation at 37°C using two different methods: 1) culture directly in a fluorescence plate reader.
and 2) culture in 96-well plates with incubation in a shaker and cell fluorescence analyzed by flow cytometry.

The fluorescence plate reader was a Tecan Infinite 200 (Tecan, Switzerland). Cultures were performed directly in the reader with 5 mm orbital shaking for 6 h after induction with hourly measurements of fluorescence and OD600. GFP fluorescence was measured with an excitation wavelength (λex) of 485 nm and an emission wavelength (λem) of 535 nm with the gain set at 115 unless otherwise stated.

The flow cytometry measurements were done by first incubating the induced cultures for 4 h, centrifugation of 1 ml of culture (12,000 x g, 1 min, RT), and cell resuspension with TBAC buffer (PBS buffer containing 1 mm EDTA and 0.01% (v/v) Tween 20). The GFP fluorescence was measured in a BD AccuriTM C5 flow cytometer (San Jose, CA, United States) with a 488 nm longpass and a 533/30 nm bandpass filter set. Data were analyzed using FlowJoTM software to obtain FL median (the fluorescence median intensity) and coefficient of variation (CV) values. The CV was calculated as follows:

\[
CV(\%) = \frac{\text{standard deviation of the sample}}{\text{mean}} \times 100.
\]

**Fluorescent Measurement With Mixed E. coli Cultures**

E. coli MG1655 cells transformed with plasmids carrying either gfp or rfp were separately inoculated and grown at 37°C and 120 rpm. The cultures were diluted 100-fold in LB medium (10 ml) and incubated in 60 ml flasks until OD600 reached ~0.8. Each culture was diluted to OD600 of 0.6, and both cultures (one carrying gfp and another rfp) were mixed in equal proportion. 200 µL of the mixture was transferred to 96-well plates (Greiner 96 Flat Bottom Black Polystyrene) and increasing concentrations of quercetin in DMSO were added. Only DMSO was added to the uninduced control. The cultivation and fluorescence measurement were carried out as mentioned above. GFP fluorescence was measured with an excitation wavelength of 485 nm and an emission wavelength of 535 nm. RFP fluorescence was measured with an excitation wavelength at 540 nm and emission wavelength of 650 nm.

**Hill Fitting and Statistical Analysis**

The fluorescence data for each concentration of quercetin were fitted with the Hill function, as follows:

\[
\frac{\text{FL}}{\text{OD}_{600}} = y_0 + \frac{\beta |Q|^n}{\left(|Q|^n + K_{0.5}^n\right)}
\]

where FL/OD600 is the specific fluorescence, y0 is the basal specific fluorescence, β is the relative maximum specific fluorescence, |Q| is the quercetin concentration in µM, n is the Hill coefficient, and K0.5 is the Hill constant (half-maximal quercetin concentration, µM). The fitting was carried out using the Solver function in Microsoft Excel®. Statistical analyses were carried out using the independent two-sample t-test with the R package (R Core Team, 2020).

**RESULTS**

**Comparing the Regulation of Two Promoters Repressed by QdoR**

To construct genetic circuits responsive to quercetin, we first dissected the QdoR regulatory system of B. subtilis, isolating qdoR, qdoI, QdoR operators, and their promoters (Figure 1A). We reasssembled them with synthetic parts (constitutive promoters, a ribosome binding site, and a transcription terminator) and cloned them in plasmids to transform E. coli. The sequence of each genetic part used is provided in Supplementary Table 1. Firstly, we cloned qdoR under the control of its promoter (PqdoR) and the reporter gfp under the control of the qdoI promoter (PqdoI). Both genes have a synthetic

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**TABLE 1 | Plasmids constructed and used in this study.**

| E. coli | Relevant characteristics | References |
|---|---|---|
| TOP10 | Cloning strain | Invitrogen |
| MG1655 | E. coli K-12-derivative strain | Beattie et al. (1997); Soupene et al. (2003) |

Plasmid

- pSB1C3: High copy number plasmid for Biobricks assembly (standard RFC [10] carrying chloramphenicol resistance
- pSB1A2: High copy number plasmid for Biobricks assembly (standard RFC [10] carrying ampicillin resistance
- pFMK1: CmR, PqdoR-RBS-qdoR-T- PqdoI-RBS-gfp-T
- pFMK2: CmR, PqdoR-RBS-qdoR-T- PqdoI-RBS-gfp-T
- pFMK3: CmR, PqdoI-RBS-qdoR-T- PqdoI-RBS-gfp-T
- pFMK4: CmR, PqdoI-RBS-qdoR-T- PqdoI-RBS-gfp-T
- pFMK5: CmR, PqdoI-RBS-qdoR-T- PqdoI-RBS-gfp-T
- pFMK6: CmR, PqdoI-RBS-qdoR-T- PqdoI-RBS-gfp-T
- pFMK7: CmR, PqdoI-RBS-qdoR-T- PqdoI-RBS-gfp-T
- pFMK8: CmR, PqdoI-RBS-qdoR-T- PqdoI-RBS-rfp-T
- pFMK9: CmR, PqdoI-RBS-qdoR-T- PqdoI-RBS-rfp-T
- pFMK10: AmpR, PqdoI-RBS-gfp-T
- pFMK11: AmpR, PqdoI-RBS-gfp-T
- pFMK12: AmpR, PqdoI-RBS-qdoR-T- PqdoI-RBS-gfp-T

Pfi means the subscript refers to the promoter sequence; RBS, Bio0034 BioBrick code; T, Bio015 BioBrick code; gfp, gene expressing the GFPmut3b variant of GFP; rfp, gene expressing the mRFP variant of DsRed.
RBS (B0034) at their 5′ flank and a double terminator (B0015) at 3′ flank. Quercetin induced GFP expression in *E. coli* (Figure 1B), demonstrating that the *P*<sup>qdoR</sup>-*qdoR*-*P*<sup>qdoI</sup>-*gfp* circuit was responsive between 20 and 80 µM quercetin. The quercetin induction increased the specific fluorescence intensity (Fluorescence/OD<sub>600</sub>) 33-fold, representing a 4.7-fold increase in the lowest to highest reporter expression compared to a previous quercetin biosensor in *E. coli* (Siedler et al., 2014). The Hill coefficient for *P*<sup>qdoR</sup>-*qdoR*-*P*<sup>qdoI</sup>-*gfp* circuit was 2.54 (R<sup>2</sup> = 0.97), displaying a cooperative and ultrasensitive response (Bradley et al., 2016).
As reported by Hirooka (2007), the qdoI promoter has two QdoR operator sites, whereas only one operator was identified upstream qdoR. In turn, to check whether a more repressed state with two operators could amplify the output upon quercetin induction, we rearranged the circuit components putting qdoR under the control of the qdoI promoter (Figure 1C). For the sake of clarity, hereafter, we shall refer to input as the quercetin concentration added to the system and output as the fluorescence level generated by GFP or RFP expression.

Although quercetin induced GFP expression, a high basal fluorescence was measured in uninduced E. coli, indicating leakage of the P_qdoI controlling the reporter. The basal fluorescence was 130 times higher than that in the negative regulated P_qdoR-qdoR-P_gfp circuit. The specific fluorescence increased only 1.8-fold from 10 to 80 µM quercetin. The Hill coefficient for this plot was 2.61; however, the data from this circuit did not fit so well the sigmoidal function ($R^2 = 0.89$).

The P_qdoI controlling qdoR should be more repressed by QdoR than P_qdoR, which decreased the QdoR concentration in E. coli and made the reporter module a little repressed. In contrast, P_qdoR was unable to control the reporter module, even when we put qdoR under the control of a strong constitutive promoter, J23110, as the GFP expression controlled by P_qdoR was completely derepressed and non-responsive to quercetin (Supplementary Figure S2). Even adding quercetin above 100 µM to E. coli with the P_qdoR-qdoR-P_gfp circuit, we did not reach the maximum specific fluorescence produced by the P_qdoI-qdoR-P_gfp circuit. Likely, the circuit P_qdoR-qdoR-P_gfp forms negative feedback induced by quercetin, resisting to express more GFP as more quercetin is added. On the other hand, the P_qdoR-qdoR-P_gfp circuit leaves the reporter module almost completely unpressed due to low QdoR expression. In agreement, the basal specific fluorescence of P_qdoI-qdoR-P_gfp is high (~4 × 10^5 AU/OD_600). Of note, quercetin uptake has already been studied in E. coli W3110, showing that in a solution with 69 µM quercetin, the intracellular quercetin concentration was stable at ~2.5 µM from 2 to 6 h of incubation (Said et al., 2016). We did not notice the growth effect in E. coli MG1655 with quercetin from 10 to 100 (data not shown).

In summary, two operator boxes, as given by the qdoI promoter, are necessary to increase repression and control the reporter module. On the other hand, the weak repression in the qdoR promoter is necessary to maintain the QdoR levels to control both regulatory and reporter modules. Moreover, manipulating the QdoR expression would make it possible to tune circuits to give different outputs.

The Constitutive Expression of QdoR With Synthetic Promoters Creates Circuits That Generate Distinct Outputs for the Same Input

We then investigated whether we could get staggered outputs in circuits with the level of QdoR adjusted using constitutive promoters of medium and low transcription strength. By staggered outputs, we mean that the maximum GFP expression of these circuits will reach intermediate values, smaller than those for the unpressed circuit (P_qdoI-gfp). The circuits were designed with non-feedback regulation since the QdoR expression does not depend on the quercetin concentration. Similar arrangements expressing the TetR repressor constitutively were applied to evaluate the output levels and noise generated in E. coli (Dublanche et al., 2006) and Saccharomyces cerevisiae (Nevozhay et al., 2009). Although negative autoregulation has been used to adjust inducer-output response curves and reduce noise in expression (Becskei and Serrano, 2000; Dublanche et al., 2006; Nevozhay et al., 2009), if we apply a circuit where QdoR represses its own expression and the reporter simultaneously, we will obtain a closed loop giving a single input-output response upon induction. On the other hand, by analogy with Ohm’s law (V = I × R), if we design circuits with different resistances (QdoR expression, analogous to the resistance R), they should respond with different outputs (GFP expression, analogous to the current I) to the same input (quercetin concentration added, analogous to the applied voltage). To verify this hypothesis, we put qdoR under the control of five promoters with reference relative expression strengths ranging from 0.10 to 0.33 (Figure 2A, Supplementary Figure S1). The relative strengths were reported previously by J. Christopher Anderson and are available at the iGEM repository dataset (http://parts.igem.org/Promoters/Catalog/Anderson). However, as we cannot guarantee that our experimental conditions were the same as those used by J. Christopher Anderson, we determined the strengths of the synthetic promoters and the P_qdoI and P_qdoR promoters (Supplementary Figure S1). All the circuits detected a minimum concentration of 20 µM quercetin, and the highest fluorescence was reached with 80–100 µM (Figure 2B). The circuit with the lowest resistance (J23114-qdoR) had a 3.5-fold increase in the dynamic range with 80 µM of inducer and the highest cooperativity ($n = 3.79$, Table 2). The maximum output decreased with increasing resistance in J23115-qdoR and J23116-qdoR circuits, but they had lower reporter leakage in the uninduced state, comparing y0 values in Table 2. Although the output gain between the uninduced and induced states was 15-fold, the cooperativity was reduced (J23115-qdoR, $n = 2.4$; J23116-qdoR, $n = 2.4$). However, in J23105-qdoR and J23110-qdoR, where the promoter controlling the circuit had in our experimental conditions 6 and 37% of the strength of a sigma 70 consensus promoter in E. coli (J23119), respectively (Supplementary Figure S1), although the circuit was still inducible, it tended to be locked into a non-activable state; 100 µM of inducer gave only 18% of the maximum output measured for the less resistive circuit with the lowest qdoR expression, J23114-qdoR (Figures 2B, C). The medium resistance circuits (J23115-and J23116-qdoR) gave maximum outputs that were around 3-fold higher than those obtained with the higher resistance circuits (J23110-and J23105-qdoR). The cooperativity was also severely reduced ($n = 1.4$). The J23119-qdoR circuit showed a low derepression even above 50 µM quercetin (the maximum output was $0.8 	imes 10^5/OD_{600}$, Supplementary Figure S3).

In summary, non-feedback circuits based on QdoR can exploit constitutive promoters with transcription forces less than 25% of the strongest consensus promoter, J23119. Above that, the amount
of QdoR per cell would become excessive, blocking all QdoR operator sites in $P_{qdoI}$, even with a high inducer concentration.

**Circuits With High Resistance Are Prone to Be Noisy**

Variation in transcription is a significant factor in generating gene expression noise (Raj and van Oudenaarden, 2008). Considering that the constitutive promoters controlling $qdoR$ used in this work have a wide range of transcription strength, it is likely that some of the non-feedback circuits expressing QdoR would be noisy. We evaluated gene expression noise with the coefficient of variation (CV), given by the ratio of the standard deviation to the mean. The CV values were obtained by flow cytometry analysis of the fluorescence intensity of $E. coli$ cells carrying each expression circuit. For the circuit with negative feedback by QdoR ($P_{qdoR-qdoR}$), the CV increased 2.5-fold between the uninduced and the fully induced state with 80 µM of quercetin (Figure 3A).
same increase in CV was also obtained for the circuit with higher resistance (J23110-qdoR) when quercetin was added to the system. In the same way, the CV in the induced state was twice the CV in the uninduced state. Interestingly, for the lower resistance circuit (J23114-qdoR), the CV decreased upon induction, reducing 2.3-fold with 50 µM of quercetin.

We extended the analysis to the other circuits and found that for the circuits with higher resistance to derepress GFP expression (J23110-qdoR and J23105-qdoR), the CV increased from the uninduced to the fully induced state. For the medium resistance circuits (J23115-qdoR and J23116-qdoR), induction reduced the CV by about 3-fold (Figure 3B, Supplementary Figure S4).

Under high repression (Figure 3C), when reporter expression is induced, many QdoR molecules can still bind many operators and block transcription of gfp. Therefore, a small number of cells within the clonal population can reach a derepression level sufficient to express some GFP. An initial highly repressed state makes the population more homogeneous but making it noisy when the input comes. Under low repression (Figure 3D), the scenario is the reverse; there are too few QdoR molecules to repress GFP expression entirely in the uninduced state, which leads to noise. Induction increases the homogeneity of the population since there are sufficient quercetin molecules to bind most of the QdoR molecules. This behavior is what Ozbudak et al. referred to as translational burst, which occurs when a cell population has a low transcriptional rate but a high translational rate (Ozbudak et al., 2002).

### DISCUSSION

In this work, we dissected and reassembled the QdoR system of *B. subtilis* in *E. coli* to engineer genetic circuits sensitive to quercetin. Such circuits have different output values to the same input controlling expression of QdoR. We correlated the strength of promoters used and the response curves of GFP expression. We highlight some differences of our circuit to this one reported by Siedler et al. (Siedler et al., 2014). We inserted the synthetic RBS B0034 upstream of qdoR and gfp. The B0034 RBS is a 12 bp sequence of medium strength in protein synthesis compared to the strong RBS used in the Elowitz and Leibler repressilator (Elowitz and Leibler, 2000). Siedler et al. have cloned the native promoters of the qdoR and qdoI, including the native RBS, which are possibly not recognized, and B0034 by *E. coli* ribosomes. In addition, we noted that in the circuit by Siedler et al., according to the sequence of p441-QdoR, there is no transcriptional terminator additionally inserted downstream of qdoR and upstream of the qdoI promoter. Eventually, the double terminator that we inserted downstream qdoR isolated it from the reporter module (P_qdoR-gfp), preventing any transcriptional interference of P_qdoR over P_qdoI. In addition, it should be noted that p441-QdoR used by Siedler et al. is a pSEVA441 derivative plasmid with the pRO1600/ColE1 origin of replication (Silva-Rocha et al., 2013). ColE1 origin was reported generating 50–70 copies/cell of *E. coli* (Lutz and Bujard, 1997), while the pUC origin as in our plasmids generated ~500-700 copies/cell. Therefore, the direct comparison of our data with those of Siedler et al. should consider a possible effect of gene dosage on GFP expression. However, as we used the minimum and maximum level of specific fluorescence of both circuits to compare performance, any effect of gene dosage is likely to be already implicit.

A similar dissection and reassembling approach was previously reported in which the FdeR native architecture of *Herbaspirillum seropedicae* was used to construct biosensors responsive to naringenin, with transcriptional factor expression control through different in silico designed RBS (De Paepe et al., 2018). However, a linear correlation of the translation initiation rates of the RBS that controlled FdeR synthesis with either the maximum output or the operational range was not found. Compared with our results, varying the strength of the promoter controlling the transcription of *fdeR* could also be

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**TABLE 2** Data fitting for the parameters of the circuits induced by quercetin using Hill function.

| Circuit | $y_0$ ($\times 10^3$) | $b$ ($\times 10^{-3}$) | $K_{0.5}$ (µM) | $n$ | $R^2$ |
|---------|---------------------|---------------------|-----------------|-----|-------|
| $P_{\text{qdoI}}$-qdoR-$P_{\text{qdoR}}$-gfp | 0.03 | 1.14 | 51.98 | 2.54 | 0.97 |
| $P_{\text{qdoI}}$-qdoR-$P_{\text{qdoR}}$-gfp | 4.20 | 6.77 | 45.61 | 2.61 | 0.89 |
| J23114-qdoR-$P_{\text{qdoI}}$-gfp | 1.94 | 7.48 | 37.60 | 3.79 | 0.99 |
| J23115-qdoR-$P_{\text{qdoI}}$-gfp | 0.23 | 4.74 | 51.30 | 2.09 | 0.99 |
| J23116-qdoR-$P_{\text{qdoI}}$-gfp | 0.12 | 3.82 | 63.48 | 2.37 | 0.98 |
| J23105-qdoR-$P_{\text{qdoI}}$-gfp | 0.11 | 1.74 | 56.16 | 2.90 | 0.96 |
| J23110-qdoR-$P_{\text{qdoI}}$-gfp | 0.15 | 1.33 | 82.72 | 1.45 | 0.99 |

$y_0$, basal output; $b$, maximum output; $K_{0.5}$, quercetin concentration to reach half of the maximum output; $n$, Hill coefficient; $R^2$, coefficient of determination.
FIGURE 3 | The QdoR expression level affects the amount of noise upon quercetin induction. (A) The coefficient of variation (CV) was calculated from the FL values measured by flow cytometry ($\lambda_{\text{ex}} = 488 \text{ nm, } \lambda_{\text{em}} = 530 \text{ nm}$). Three circuits controlling gfp expression were induced with increasing quercetin concentrations: $P_{\text{qdoR}}$-qdoR (qdoR expression controlled by the minimal qdoR promoter) and J23110-qdoR and J23114-qdoR (qdoR is expressed constitutively by the J23110 and J23114 promoters, respectively). The experiment was conducted with biological triplicates. (B) The CV of GFP expression in uninduced and induced E. coli cultures was correlated with the circuits depicted in Figure 2A; all these circuits had gfp as a reporter. The J23114-gfp construct has the J23114 constitutive promoter controlling the expression of gfp. The $P_{\text{qdoI}}$-gfp construct has the non-repressed qdoI promoter controlling gfp expression. Note that both circuits without QdoR repression do not have considerable variations on CV values upon the addition of quercetin. (C, D) Illustrative representation of the effect of QdoR expression on the CV values of GFP expression. Two states are considered in either high QdoR expression (J23110-qdoR) or low QdoR expression (J23114-qdoR): in light red, the uninduced repressed circuit, in light green, the circuit induced with 100 µM quercetin. J23110-qdoR and J23114-qdoR represent the regulatory modules. Below them, the possibilities of outputs to the reporter modules are depicted. White bacteria are completely repressed by QdoR with no GFP expression, while green bacteria have some GFP expression level that increases when quercetin is added. Note that the number of QdoR is high when J23110 controls the circuit, leading to derepression resistance when quercetin is added and an increase in noise (high CV amplitude in counts against FL plot). The cytometric profiles (counts vs. fluorescence intensity) to each circuit at the uninduced and induced states are provided in Supplementary Figure S4 and Supplementary Table S3. Statistical significance of the comparison for each circuit in uninduced and induced states is shown as $p$-values* $\leq 0.05$, ** $p$-value $\leq 0.01$, *** $p$-value $\leq 0.001$ (independent two-sample t-test). The statistical comparisons between all circuits are provided in Supplementary Table S2. The experiments were conducted with biological triplicates. After induction, the fluorescence of each replicate was measured once at specified times. The experiments were conducted with biological triplicates. After 4 h of induction, the fluorescence of each replicate was measured once. The error bars represent the standard deviation.
an interesting alternative to tune both maximum output and operational range.

By combining the native elements of the native QdoR system with synthetic elements, we designed circuits with varied output values and different noise behavior in the presence or absence of an inducer. We determined that noise is minimal when the repressor QdoR is either mostly free or mostly bound to the inducer. On the other hand, when the level of free QdoR is similar to the level of QdoR bound to quercetin, noise is likely to increase due to transcriptional bursts when a gene is transcribed in a pulse. Negative feedback loops (Savageau, 1974; Becskel and Serrano, 2000) can minimize noise. For example, a circuit with TetR repressing itself and a reporter gene decreased noise compared to a circuit expressing TetR constitutively at high levels, as in the J23110-qdoR circuit.

The circuits controlled by QdoR were constructed in high copy number plasmids and showed good reproducibility and stability. Further optimization of the circuits, including reduction of leakiness and increases in the sensitivity and the dynamic range, might be necessary depending on their application. It would also be possible to engineer circuits that have response delays (Hooshangi et al., 2005) or that act as inverters, with high input producing low output. Our research group has a long-term goal of developing gene circuits for plant-bacteria interaction. Such circuits will benefit studies on chemical communication between beneficial rhizobacteria and plants (Pini et al., 2017; Poole, 2017) and aid in designing potential biotech applications. In this sense, the benchmarking of minimal parts, such as the PqdoR and PqdoI promoters in circuits controlled by quercetin, will be fundamental for bioengineering efficient circuits exploring

**FIGURE 4** The same inducer concentration gives different outputs in a mixed culture of E. coli strains. (A) Strain 1, E. coli MG1655 carrying the circuit J23114-qdoR-Pqdol-gfp was mixed with strain 2, E. coli MG1655 with J23110-qdoR-Pqdol-rfp, keeping an equal proportion of both strains. The mixed culture was induced with different concentrations of quercetin, and fluorescence (FL) was measured using the following combinations of emission (λem) and excitation (λex) wavelengths: 485 nm (λem) and 535 nm (λex) for green fluorescence measurements and 540 nm (λem) and 650 nm (λex) for red fluorescence measurements. Note that the graph in A has a break in the y-axis to show the data plotted for strain 2. (B) To rule out any effect of the reporter itself on the results, we measured the outputs as in A, but with the following configuration: strain 1, E. coli MG1655 carrying J23114-qdoR-Pqdol-rfp, and strain 2, E. coli MG1655 carrying J23110-qdoR-Pqdol-gfp. Q, quercetin. The experiments were conducted with biological triplicates. After induction, the fluorescence of each replicate was measured once at specified times. The error bars represent the standard deviation.
plant-bacteria communication. For instance, circuits controlled by flavonoids can be applied to control gene expression in bacteria associated with plants, as shown in bacteria expressing genes of the nif (nitrogen fixation) cluster when associated with cereals (Ryu et al., 2020).

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding author.

**AUTHOR CONTRIBUTIONS**

FK, BM, ES, and MS conceptualized the circuits and experimental strategy. FK and BM performed the experiments. FK, BM, FP, ES, and MS analyzed data. FK, BM, FP, ES, and MS wrote the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2021.730967/full#supplementary-material
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