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Multistage Regulator Based on Tandem Promoters and CRISPR/Cas

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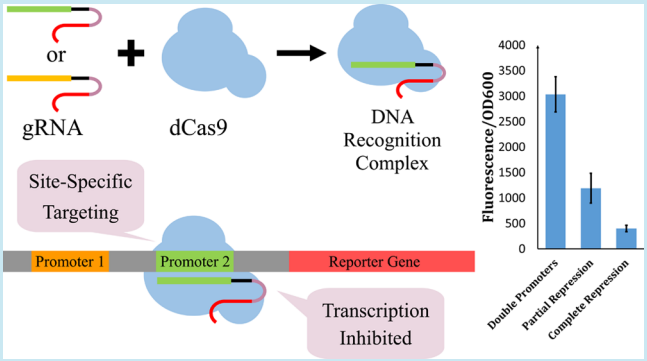
ABSTRACT: Accurately controlling expression of target genes between several designed levels is essential for low-noise gene network and dynamic range of gene expression. However, such manipulations have been hard to achieve due to technical limitations. Based on tandem promoters and CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR-associated) system, we constructed a multistage regulator that could stably regulate the expression of the reporter gene on three levels, with more than 2-fold difference between each of them. Our findings provide novel insights into constructing a more powerful gene regulation system.



Synthetic biology and metabolic engineering require more efficient applications of each gene element, for contribution to construction of low-noise gene networks or increased dynamic range of gene expression. While previous work has focused on single promoters, challenges remain with respect to multistage control of gene regulation.

There are two main aspects of multistage regulation: (1) a limited number of different single promoters can be combined in a linear tandem manner to form a synthetic promoter of a much higher relative activity threshold,[1] and (2) considering that expression levels of such tightly controlled synthetic promoters are determined by the subpromoters they are composed of, once they are specifically turned on or off, a low-noise mechanism becomes available, enabling stable and diverse gene expression levels as desired.

Therefore, stable, accurate, and orthogonal machinery was our first choice for site-specific targeting. We chose the Cas9 protein that belongs to the CRISPR/Cas system and has been applied in fields such as genome editing[2] and gene activation/repression.[3,4] Cas9 is a DNA endonuclease, which needs a guide RNA (gRNA) and protospacer-adjacent motif (PAM) region (NGG) to bind to the target sequence through base pair complementation. Since binding efficiency of Cas9 is the only function needed in this study, the double mutant D10A/H840A resulting in a catalytically "dead" Cas9 (dCas9) was used.[2]

Here, we chose three different promoters from the Anderson promoters library,[5] synthesized several double promoters, and engineered the CRISPR/Cas system to specifically turn off one or two of the subpromoters. As the silencing part of dCas9 is determined by its gRNA, orthogonality and accuracy of expression control can be precisely obtained by site-specific targeting (Figure 1), turning off the subpromoters of tandem promoters, largely diversifying and widening the available expression levels and range.

RESULTS

Synthetic Tandem Promoter Has Higher Expression Strength. Three promoters, J23102, J23106, and J23116 of different strengths (0.86, 0.47, and 0.16, respectively, compared to promoter J23100), were used to construct double promoters[5] (Figure 2a). Strengths of these tandem promoters were higher than the downstream subpromoter, which implies that synthetic promoters can be used more broadly for further engineering. For example, combination J23106-102 has a higher strength than single promoter J23102 and J23106 (Figure 2b).

CRISPR/dCas9 Repression System Widens the Range of Expression Levels. Two separate gRNAs were designed to target J23106 and J23116 after assembly with dCas9 protein. Once bound to the sequence of interest, they block the initiation process of RNA polymerases. When the upstream promoter (J23106 in Figure 3) is bound by gRNA-dCas9 silencing complex, it is blocked, resulting in a midlevel expression of RFP reporter gene. When the complex binds to the downstream promoter (J23116 in Figure 3), expression was repressed more significantly, at about a 7.5 times range.

Another double promoter, J23106-102, as shown in Figure 2b, was also tested and showed similar expression patterns (data shown in http://2013.igem.org/Team:WHU-China). Our experiment shows that dCas9-gRNA system blocks about 75% expression of the target promoter, similar to previous results.[2]

DISCUSSION

Compared to traditional chemical-induced promoters, our multistage promoter is not only relatively more accurate to

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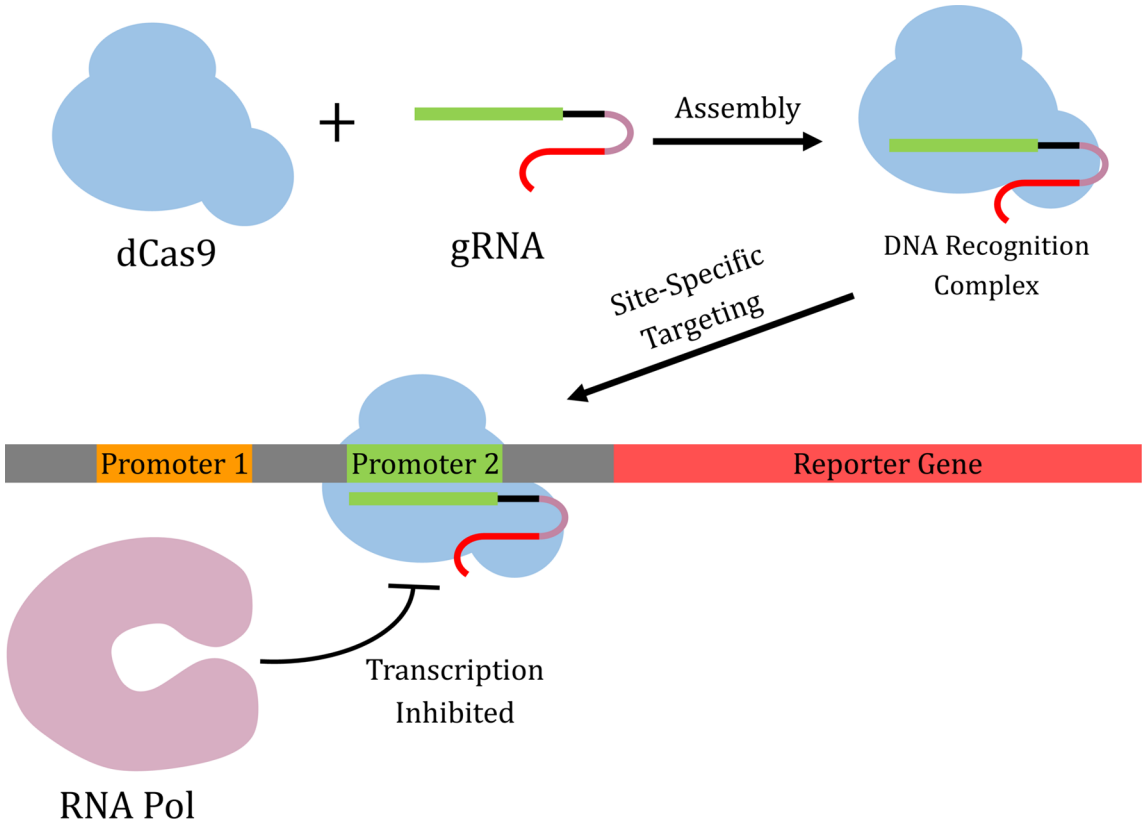

Figure 1. Mechanism of the multistage regulator. The dCas9 protein (blue) and sequence-specific guide RNA (gRNA) are coexpressed to assemble a DNA recognition complex that targets the specific site of the tandem promoters. After binding, the dCas9protein inhibits transcription by blocking the binding or elongation of the RNA polymerase.

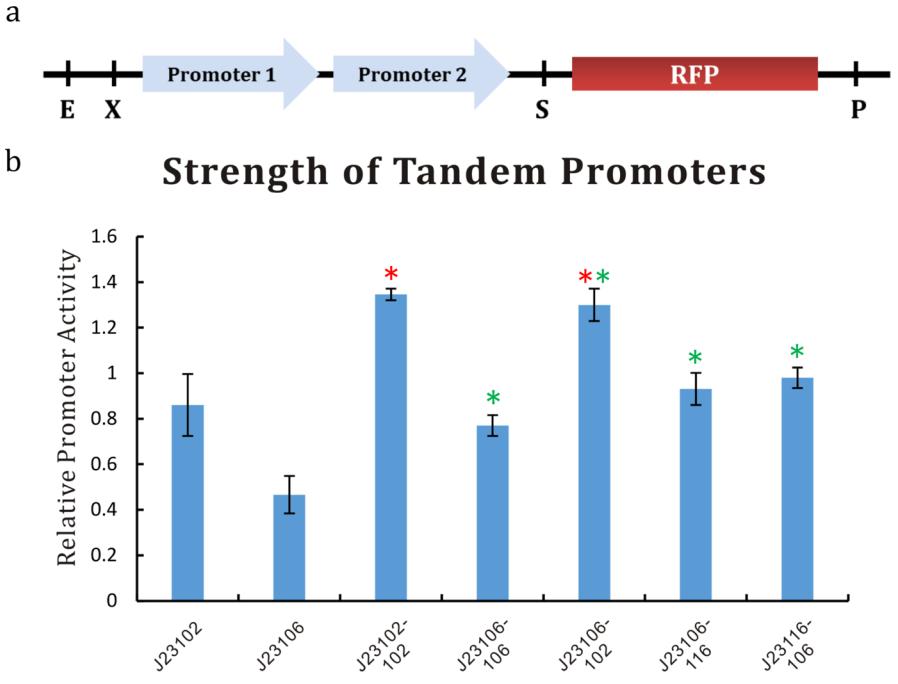

Figure 2. Strength of different tandem promoters. (a) The tandem promoter system constructed contains two promoters selected from J23102, J23106, and J23116. Red fluorescent protein (RFP) was used as the reporter gene. (b) The strengths of the tandem promoter systems were measured by detecting the relative fluorescence intensity of *E. coli* cultures transformed by tandem promoter plasmids. *E. coli* cells expressing RFP were excited at 584 nm, and the fluorescence emission was detected at 607 nm. Promoters J23102 and J23106 were used as a dual standard control system to indicate that the transcription strength of these tandem promoters further improve the accuracy. Error bars indicate the standard error of mean for three independent biological replicates. Red asterisks (*) on each bar indicate statistically significant changes in RFP expression relative to J23102 control; green asterisks on each bar indicate statistically significant changes in RFP expression in samples relative to J23106 control (t test, p-value < 0.05).

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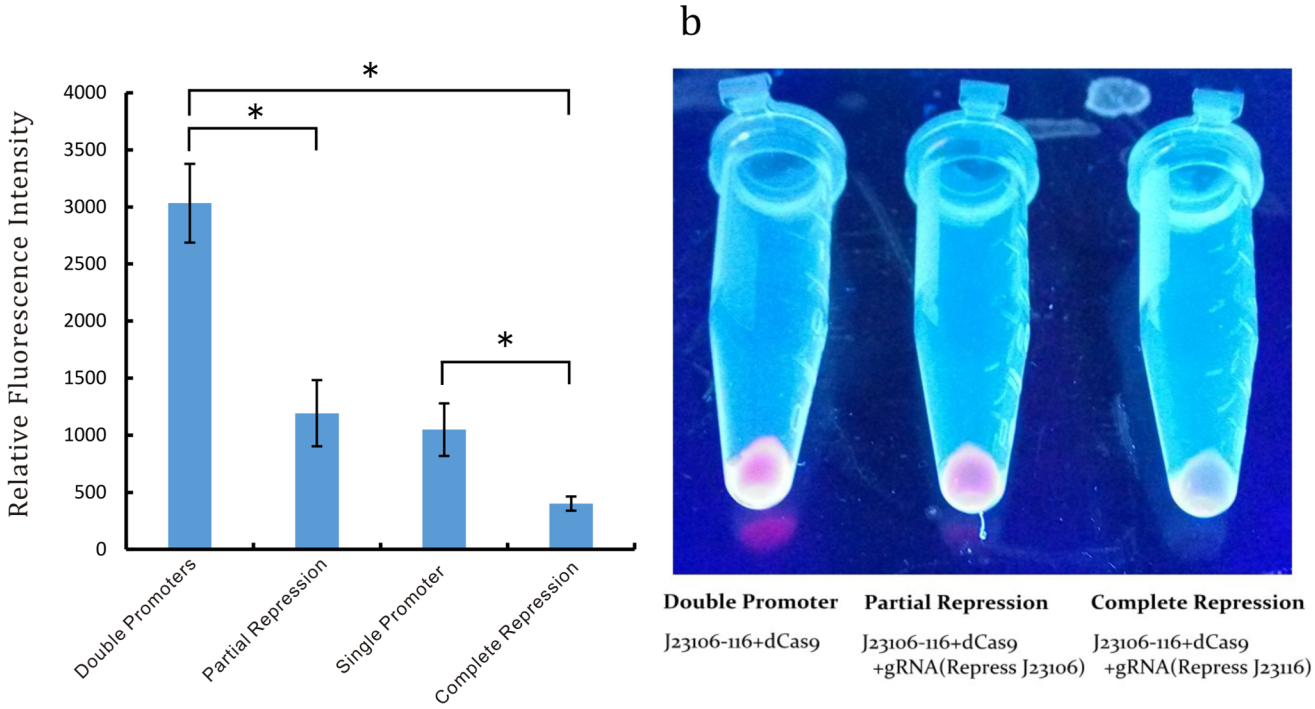

Figure 3. Different expression levels of RFP reporter gene with/without artificial repression. (a) Site-specific gRNAs were coexpressed with dCas9 protein. Strength changes of RFP reporter gene were measured via fluorescence assay. Double promoters refers to promoter combinations J23106 and J23116, upstream to downstream. Partial and complete repression was performed on double promoters with J23106 and J23116 targeted, respectively. Single promoter J23116, as well as double promoters, were used as controls (*t* test, *p*-value < 0.05). (b) Equal amount of *E. coli* sediments were collected in microcentrifuge tubes and exposed under UV light. The less red the sediments, the stronger the repression effect.

use and resistant to cellular noise, but also provides a larger range of expression,as shown above. Therefore, our multistage promoter provides a more robust gene element for biological analog computing networks, and a much more reliable tool for metabolic engineering.

This multistage regulator can be further improved in two ways. First, the orthogonality of the system can be improved by inserting special targeting barcode to spaces between subpromoters. Second, activators can be brought in to activate specific subpromoters, leading to a broader expression range. Thus, our multistage regulator is easily adaptable with several potential applications.

METHODS

Plasmids and Strains. dCas9 was provided by Addgene. BBa_J13002 (pTetR and B0034 chosen from 2012 iGEM Distribution Kit) was added upstream to the coding sequence of dCas9 to make the functional dCas9 protein on pSB1A2 and pSB1C3 (standard vectors chosen from the kit, also shown at http://2013.igem.org/Team:WHU-China). Cloning strains, *Escherichia coli* DH5α, were cultured in LB medium overnight at 37 °C. Ligation concerned with dCas9 gene was preformed via Gibson assembly.

gRNA Repression. Site-specific gRNAs, including N20 and thedCas9 handle and terminator, were synthesized by Sangon Biotech. N20 of R1-TGACAGCTAGCTCAGTCCTA and R2-GCGGCCGCTTCTAGAGTTTA were designed to target J23106 and J23116, respectively. The remanining sequences of the dCas9 handle and terminator were the same as those described in the Zhang paper.[2]

Fluorescence Measurement and Analysis. OD_{600} and absolute fluorescence intensity (AFI) were measured via automatic microplate reader. The relative fluorescence intensity (RFI) was calculated using the formula, RFI = AFI/OD_{600}. Three independent biological samples were used to calculate the mean and standard error for each data point. Subsequent analysis was performed in Microsoft Excel 2013.

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Author Contributions

†H.J., T.L., and Z.W. contributed equally. C.Z.H., H.J., L.Y., Y.L., Y.Z., J.W., Z.X., and Y.C. conceived and designed the experiments. H.J., T.L., Z.W., L.Y., Y.L., L.T., and S.L. performed the experiments. Z.H., H.J., and Z.W. analyzed the data. Z.H., H.J., Z.W., T.L., L.Y., Y.L., and Z.X. wrote the paper.

Notes

The authors declare no competing financial interest.

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