Gene knockdown by structure defined single-stem loop small non-coding RNAs with programmable regulatory activities

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\textbf{ABSTRACT}

Gene regulation by \textit{trans}-acting small RNAs (sRNAs) has considerable advantages over other gene regulation strategies. However, synthetic sRNAs mainly take natural sRNAs (MicC or SgrS) as backbones and comprise three functional elements folding into two or more stem-loop structures: an mRNA base pairing region, an Hfq-binding structure, and a rho-independent terminator. Due to limited numbers of natural sRNAs and complicated backbone structures, synthetic sRNAs suffer from low activity programmability and poor structural modularity. Moreover, natural sRNA backbone sequences may increase the possibility of unwanted recombination. Here, we present a bottom-up approach for creating structure defined single-stem loop small non-coding RNAs (ssl-sRNAs), which contain a standardized scaffold of a 7 bp-stem-4 nt-loop-polyU-tail and a 24 nt basing pairing region covering the first eight codons. Particularly, ssl-sRNA requires no independent Hfq-binding structure, as the polyU tail fulfills the roles of binding Hfq. A thermodynamic-based scoring model and a web server sslRNAD (http://www.kangzlab.cn/) were developed for automated design of ssl-sRNAs with well-defined structures and programmable activities. ssl-sRNAs displayed weak polar effects when regulating polycistronic mRNAs. The ssl-sRNA designed by sslRNA showed regulatory activities in both Escherichia coli and Bacillus subtilis. A streamlined workflow was developed for the construction of customized ssl-sRNA and ssl-sRNA libraries. As examples, the E. coli cell morphology was easily modified and new target genes of ergothioneine biosynthesis were quickly identified with ssl-sRNAs. ssl-sRNA and its designer sslRNA enable researchers to rapidly design sRNAs for knocking down target genes.

1. Introduction

\textit{Trans}-acting small non-coding RNAs (sRNAs) with lengths of 50–300 nt are crucial post-transcriptional regulators which are involved in many physiological processes such as metabolic regulation, substrate transport and stress response [1–6], etc. Like RNA interference (RNAi) by small interfering RNA (siRNA), short hairpin RNA (shRNA) and bi-functional shRNA, sRNAs also act in \textit{trans} and commonly form RNA double strands with their target mRNAs to regulate gene expression by inhibiting translation or accelerating mRNA decay [7]. But different from siRNAs or shRNAs, sRNAs undergo no post-transcriptional processing and generally have more complicated secondary structures because of their longer sequences. It is well established that sRNAs comprised three functional elements: an mRNA base pairing region, an Hfq-binding structure, and a rho-independent terminator [8,9]. Most of the time, sRNA hybridizes with its target mRNAs with the help of RNA chaperons Hfq, ProQ or CsrA etc. [10] by either perfect or imperfect base pairing [6,11]. The RNA chaperon such as Hfq facilitate the binding of sRNA to the target mRNA [12,13]. In addition to the termination of transcription during sRNA biogenesis, the polyU tail of the terminator also plays critical roles in the binding of Hfq [8].

In 2012, Kang et al. proposed and firstly validated the application of sRNA for biotechnological purposes [14]. Therefore, synthetic sRNA regulators based on the scaffolds of natural sRNAs such as MicC, MicF,
SgrS and PrfF1 have been created for metabolic engineering applications [15–19]. Synthetic sRNAs are considered as advantageous gene repression tools when target genes are essential and cannot be knocked out or when fine downregulation is preferred to gene deletion [14,20,21]. Moreover, they allow for rapid screening of large numbers of gene candidates during metabolic pathway redesign [15,18,19].

Nowadays, synthetic sRNAs with repression activities are largely created by replacing the base-pairing region of natural sRNAs [15,16,20,22], whose Hfq-binding structures and rho-independent terminators contain two or more stem-loop structures. These complicated secondary structures may mean screening of sRNA scaffolds to create functional synthetic sRNA [15]. The major challenge is that the sRNA activities are difficult to program due to the unknown sequence-structure-activity correlations of the limited numbers of natural sRNA scaffolds. Moreover, natural sRNA backbone sequences may increase the possibility of unwanted recombination, especially when expressing multiple sRNAs.

The aim of this study is to explore sRNA rational design principles and develop a convenient tool for bottom-up design of synthetic sRNAs with programmable activities and diversified and unnatural sequences. We found rho-independent terminators (single stem-loop structure) directly connecting to a 24 nt or longer base pairing region making ssl-sRNAs function in Escherichia coli and Bacillus subtilis. We demonstrated that rho-independent terminators with a 7 bp stem, a 4 nt loop and a 7 or longer polyU tail conferred sRNA stronger repression activities than other structures. The ssl-sRNA regulating activities were fine-tuned by modulating the thermodynamics of the aforementioned stem-loop structure. ssl-sRNA scoring function and web server sslRNAD (single stem loop sRNA designer, http://www.kangzlab.cn/) were developed to computationally design ssl-sRNAs. To speed up the screening process and simplified the experimental operations, we also develop a streamlined workflow for construction and application of ssl-sRNA libraries targeting a set of genes by creating all the required ssl-sRNA expression vectors with one-pot PCR. With such a library, we identified several new target genes out of 80 candidates to regulate the biosynthesis of Ergothioneine within one week.

2. Materials and methods

2.1. Strains and plasmids

E. coli BL21 (DE3) was used to express sRNAs, green fluorescent protein (GFP), or chromosomally encoded β-galactosidase. All the plasmids and primers used in this study are listed in Table S1 and Table S2. E. coli JM109 was used as the host to construct, amplify and stock the plasmids.

To construct the plasmid pTargetF-Long_anti-gfp-sRNA, the gene cassette T7 promoter-Long_anti-gfp-sRNA (Table S1) was synthesized by GENEWIZ (Suzhou, China) and assembled with the BamHI & HindIII linearized pTargetF [23]. The sequence of the T7 promoter is 5′-AATATTTGACGACTCTAGATG-3′. Using pTargetF-Long_anti-gfp-sRNA A as the template, the linear forms of the plasmids pTargetF-Long_Control-sRNA, pTargetF-Middle_anti-gfp-sRNA, pTargetF-Short_anti-gfp-sRNA, pTargetF-M7_anti-gfp-sRNA to pTargetF-M7 anti-gfp-sRNA, pTargetF-GadY_anti-gfp-sRNA, pTargetF-CT_anti-gfp-sRNA, and pTargetF-T7_anti-gfp-sRNA were amplified by PCR with the corresponding sense and antisense primers containing the designated sRNA sequences and homog enous sequences (Table S2). The PCR products carrying primer-confoned homogenous 5′ and 3′ terminal (Table S2) were transformed into E. coli JM109 after purification and self-cyclized by the endogenous DNA recombinases of E. coli.

In the same way, the plasmids pTargetF-Middle_Control-sRNA, pTargetF-Short_Control-sRNA, pTargetF-M7_Control-sRNA, pTargetF-GadY_Control-sRNA, pTargetF-CT_Control-sRNA, and pTargetF-T7_Control-sRNA were constructed using pTargetF-Long_Control-sRNA as the template. The plasmids pTargetF-GadY-Variant1_anti-gfp-sRNA to pTargetF-GadY-Variant10_anti-gfp-sRNA, pTargetF-GadY-Variant3_M1_anti-gfp-sRNA to pTargetF-GadY-Variant3_M5_anti-gfp-sRNA were constructed using pTargetF-GadY_anti-gfp-sRNA as the template. The plasmids pTargetF-M7_anti-lacZ-sRNA, pTargetF-Rstem1_V3loop_anti-gfp-sRNA, pTargetF-Rstem2_V3loop_anti-gfp-sRNA, pTargetF-Rstem3_V3loop_anti-gfp-sRNA, pTargetF-Vstem4_V3loop_anti-gfp-sRNA, pTargetF-Vstem_M7loop_anti-gfp-sRNA, pTargetF-Random1_anti-gfp-sRNA to pTargetF-Random4_anti-gfp-sRNA, pTargetF-DeNovo_m7_anti-gfp-sRNA to pTargetF-DeNovo_m7_anti-gfp-sRNA was synthesized by plasmid pTargetF-M7 Control-sRNA as the template. The designated sense and antisequence primers are also listed in Table S2.

The plasmids carrying sslRNA designed ssl-sRNAs targeting ftsZ genes or the 80 genes listed in Fig. 5A were constructed following the workflow depicted in Fig. 4E or Fig. 6B. The ssl-sRNAs sequences and the interfaced promoters were all included in the automatically designed with sslRNA. The plasmids were applied to two-pair-primer PCR using pTargetF as template (Fig. 4E). The sequences of the recombinant plasmids were all confirmed by Sanger sequencing.

The plasmid pCOLADuet-gfp was constructed by insert the Shine-Dalgarno sequence ‘AAGGAGGAAAAATAT’ and the gfp coding region between the restriction sites of BgII and XhoI. The plasmid pCOLADuet-gfp-BhepIII was constructed by insert the Shine–Dalgarno sequence ‘AAGGAGGAAAAATAT’ and the BhepIII gene [24] between the restriction sites of Bsp143I and BglII.

2.2. Medium and cultivation

All strains were routinely cultivated in Luria Broth (LB) (tryptone 10 g/L, NaCl 10 g/L, yeast extract 5 g/L) or on LB agar at 37 °C. To maintain the stability of pCOLADuet-1 or its derivatives and pRSF-egtBCDE (derived from pRSFDuet-1), 50 µg/mL kanamycin was supplemented. To maintain the stability of pTargetF and its derivatives, 50 µg/mL spectinomycin was supplemented. When necessary, 0.1 mM isopropyl β-D-thiogalactoside (IPTG) was added to induce the expression of the T7 promoter (pTargetF and pCOLADuet-1, by turning on the expression of T7 RNA polymerase) and chromosomal lac promoter in E. coli BL21 (DE3).

The ergothioneine-producing E. coli strains harboring ssl-sRNA expression vectors were cultivated in M9Y minimal medium (amino acids were excluded) [25] supplemented with 20 µg/L FeSO4·7H2O and selective antibiotics kanamycin and spectinomycin. The strains were subsequently cultured at 37 °C to synthesize ergothioneine. For primary screening, the M9Y minimal medium was loaded into 24 well plate to 1.5 mL and inoculated with single colonies of the transforms of the ssl-sRNA library. After 3 h cultivation at 37 °C, IPTG were then added to the final concentration of 0.2 mM and the plates were subsequently cultured at 30 °C for 32 h to synthesize ergothioneine. For batch fermentation of ergothioneine, the M9Y minimal medium was loaded into 250 mL shake flask to 25 mL and inoculated with overnight LB seed culture of the representative transforms to an initial OD600 of 0.1. IPTG was supplemented to 0.2 mM final concentration 3 h after inoculation to induce the expression of etcBCDE operon and ssl-sRNAs. At time point 72 h, samples were collected to measure the ergothioneine concentrations.

2.3. Fluorescence measurement and microscopy

Culture samples were collected at the designated time points and diluted appropriately to measure the fluorescence intensities and cell densities (OD600). GFP fluorescence intensity was measured by an Infinite 200 PRO plate reader with an excitation wavelength of 475 nm, an emission wavelength of 510 nm and a gain of 50. Relative
fluorescence intensity was expressed as fluorescence intensity/OD_{600} (arbitrary unit, a.u.). Cells were visualized with an Eclipse Ni-E microscope (Nikon, Tokyo, Japan) equipped with a phase-contrast microscopy module. A filter set for fluorescein [excitation filter, 465–495 nm; dichroic mirror, 505 nm; emission filter, longpass (LP) 512 nm] was used when imaging green fluorescence emitted from GFP. To visualize membrane structures, cells were stained with Nile red dissolved in DMSO and imaged with an Eclipse Ni-E microscope [excitation filter, 527.5–552.5 nm; dichroic mirror, 565 nm; emission filter, (LP) 577.5–632.5 nm]. Micrographs were processed with ImageJ [26].

2.4. β-Galactosidase activity assay

Culture samples were collected at the designated time points and diluted appropriately to measure the cell densities. Cells were collected by centrifugation at 10,000 g and subjected to β-galactosidase activity, which was expressed as a Miller unit [27]. Briefly, cells were appropriately diluted in 1 mL Z buffer and permeabilized by adding 100 μL 2.4. β-577.5–527.5. Measurement of heparinases III activities

After removing cell debris by centrifugation at 10,000 g, 200 mg/L and 400 mg/L of ergothioneine standard (100 mg/L, 200 mg/L and 400 mg/L of ergothioneine were monitored with UV absorbance at wavelength of 260 nm. During HPLC analysis, flow rate was maintained constant at 0.4 mL/min and mobile phase A (0.1% v/v formic acid) went back to 10% and mobile phase B (acetonitrile) increased from 0.6 to 1.6 and collected by centrifugation at 4 °C for approximately 4 h to an OD_{600} between 0.6 and 1.6 and collected by centrifugation at 4 °C and 10,000 × g for 15 min. Total RNA was extracted with TRIzol solubilization and extraction [28]. RNA samples were separated via electrophoresis in a 15% polycrylamide-urea gel. After transferring the RNAs from the gels to nylon membranes in ice-cold TBE buffer, UV crosslinking of the RNA and the membrane was performed (120 J, 5 min). 5S rRNA and anti-gfp sRNAs were probed by the biotin-labeled polynucleotides 5’-CTACGGGGTTTCACTTTGAGTC-3’ and 5’-ATGGTTAAGGGGAAGAACTTTT-3’, respectively. Bands on northern blots were visualized by electrochemiluminescence with FUJIFILM Super RX films.

2.5. Measurement of heparinase III activities

E. coli BL21 (DE3) cells expressing BhepIII gene were collected by centrifugation at 5,000 g after 12 h of cultivation, resuspended with 20 mM Tris–HCl buffer (pH 7.4) and lysed with sonication. Cell debris was removed by centrifugation at 10,000 g. Supernatant (50 mL) was mixed with 20 mg mL\(^{-1}\) heparin sodium salt dissolved in PBS buffer (pH 7.4) to 750 μL and incubated at 30 °C for 30 s. The heparinase III activity was determined as describe previously [24]. Enzyme activities were measured by monitoring the formation of unsaturated glucuronic/iduronic acids, which absorbance light at wavelength of 232 nm, with a molar extinction coefficient of 3800 L/mol/cm [24]. One unit of enzymes was defined as the enzyme required to produce one μmol of unsaturated glucuronic/iduronic acid within 1 min at 30 °C.

2.6. Measurement of ergothioneine concentration

Cells were pelleted by centrifugation and supernatants were pooled and mixed with equal volume acetonitrile. After removing insoluble fractions with centrifugation, the supernatants were filtered through a 0.22 μm membrane. Ergothioneine in the supernatant was measured by high-performance liquid chromatography with a Waters ACQUITY Arc HPLC system equipped with a Waters Symmetry C18 column (5 μm, 4.6 × 250 mm) and Waters 2489 UV/Visible (UV/Vis) detector. Gradient elution program was set as mobile phase B (acetonitrile) increased from 10% to 50% within 10 min after sample injection; 5 min later, mobile phase B went back to 10% and mobile phase A (0.1% v/v formic acid) increased to 90%; 10 min later, next sample injection was performed. During HPLC analysis, flow rate was maintained constant at 0.4 mL/min and column temperature was set to 30 °C. Concentrations of ergothioneine were measured with UV absorbance at wavelength of 260 nm. Calibration of the peak areas was performed with 25 mg/L, 50 mg/L, 100 mg/L, 200 mg/L and 400 mg/L of ergothioneine standard (>98% purity, Shanghai Macklin Biochemical Co., Ltd, Shanghai, China). HPLC graphs were processed with Empower Chromatography Data System.

2.7. Northern blotting

Cells were cultivated at 37 °C for approximately 4 h to an OD_{600} of 0.6 and 1.6 and collected by centrifugation at 4 °C and 10,000 × g for 15 min. Total RNA was extracted with TRIzol solubilization and extraction [28]. RNA samples were separated via electrophoresis in a 15% polycrylamide-urea gel. After transferring the RNAs from the gels to nylon membranes in ice-cold TBE buffer, UV crosslinking of the RNA and the membrane was performed (120 J, 5 min). 5S rRNA and anti-gfp sRNAs were probed by the biotin-labeled polynucleotides 5’-CTACGGGGTTTCACTTTGAGTC-3’ and 5’-ATGGTTAAGGGGAAGAACTTTT-3’, respectively. Bands on northern blots were visualized by electrochemiluminescence with FUJIFILM Super RX films.

2.8. Scoring model of the ssl-sRNA activity

To evaluate the regulatory activity of an ssl-sRNA constructed from a given core scaffold, we developed a scoring function (Formula 1) by taking the different and independent contribution factors (β) of the thermodynamic details (ΔG_{Detail}) of the decomposed secondary structure (see Fig. 4) into consideration (Formula 2). Multiple regression was performed by Formula 2 to calculate the contribution factor β applied in Formula 1. Thermodynamic details (ΔG_{Detail}) of the core scaffold used to create sRNA were calculated by RNAeval (ViennaRNA 2.4.17) [29].

\[
\text{Score of the ssl-sRNA activity} = \sum_{i=1}^{7} (\Delta G_{Detail,i} \times \beta_i) 
\]

(Formula 1)

\[
\frac{\text{Flu}}{\text{OD}_{600}} = \begin{bmatrix}
1 \\
\Delta G_{Detail,1} \\
\Delta G_{Detail,2} \\
\Delta G_{Detail,3} \\
\Delta G_{Detail,4} \\
\Delta G_{Detail,5} \\
\Delta G_{Detail,6} \\
\Delta G_{Detail,7}
\end{bmatrix} \begin{bmatrix}
\alpha \\
\beta_1 \\
\beta_2 \\
\beta_3 \\
\beta_4 \\
\beta_5 \\
\beta_6 \\
\beta_7
\end{bmatrix} 
\]

(Formula 2)

where:

- Flu/OD_{600} is the measured relative fluorescence intensity (a. u.) of GFP with the regulation of anti-gfp ssl-sRNA constructed from the given core scaffold;
- ΔG_{Detail,i} is the thermodynamic detail of the i interior loop of the given core scaffold (i = 7 means the hairpin loop);
- β_i is the regression coefficient of ΔG_{Detail,i} in Formula 2, which was further defined as the contribution factor of ΔG_{Detail,i} to the regulation activity of the investigated anti-gfp ssl-sRNA;
- α is the intercept in the regression; and
- λ is the scale factor, the default value of which is \( \frac{1}{\pi} \).

2.9. Code

sslRNAD is available for academic users and is provided as a web server which can be accessed with the following link: http://www.kanglab.cn/. The source code is publicly available on Zenodo (http://doi.org/10.5281/zenodo.6914471).
3. Results

3.1. Minimize the sRNA scaffold to a single stem-loop with a polyU tail

Natural sRNAs generally fold into complicated secondary structures and output unprogrammable regulatory activities. We sought to uncover a simple core structure, which has been reduced to essentials and by attaching any designated base pairing region to the core structure, functional sRNAs would be preferably created. To this end, artificial sRNAs were first designed to explore the sRNA modularity (Fig. 1A) by hybridizing the scaffold of two natural Spot 42 [30] and MicA [31]. The Long scaffold contains the unpaired ‘AU box (orange), the Hfq binding hairpin of Spot 42 and the rho-independent terminator of MicA. In addition, the polyU tail (blue) from MicA was extended to enhance the binding of the RNA chaperone Hfq [8, 32] (Fig. 1A). A reverse complement of the start codon ‘ATG’ and the ensuing 21 nt sequence [15] of the green fluorescence protein (gfp) gene was attached to the 5′ terminus of Long scaffold and expressed in E. coli BL21 (DE3) from a plasmid (Figs. S1A and 1B). We found the Long_anti-gfp sRNA repressed the expression of plasmid-borne gfp by approximately 70.6% (Fig. 1B and Fig. S1C).

Based on the Long scaffold, a Middle scaffold was created by deleting the unpaired ‘AU box (Fig. 1A). Middle_anti-gfp sRNA repressed gfp expression by approximately 74.3% (Fig. 1B and Fig. S1C). Further truncation of the Middle scaffold destroyed the Hfq binding hairpin of Spot 42 and liberated the paired ‘AU’ rich box, which was directly connected to the rho-independent terminator of MicA with extended polyU tail (Short scaffold, Fig. 1A). The generated Short_anti-gfp sRNA displayed almost the same repression activity (Fig. 1B and Fig. S1C). This result demonstrated that it is feasible to construct functional ssl-sRNAs, which have simplified secondary structures than natural sRNAs or previously designed synthetic sRNAs [15, 20, 33–35].

We next investigated whether the unpaired ‘AU’ rich box is essential. Four nucleotide-substitution mutants and three truncation mutants were constructed (Fig. 1C). We found that all mutants exhibited gene repression capabilities and repressed gfp expression by 71.2%–87.0% (Fig. 1D and E). Particularly, the M7 scaffold could function as a minimized scaffold (Fig. 1F). These results also indicate the nonessential role of the unpaired ‘AU’ rich box, which is consistent with the Ishikawa’s mutagenesis study on SgrS sRNA and its repression of the cognate ptsG mRNA target via imperfect base pairing [32]. Northern blot analysis confirmed that the Long_anti-gfp sRNA, Middle_anti-gfp sRNA, Short_anti-gfp ssl-sRNA and the other anti-gfp ssl-sRNAs were expressed at levels slightly lower than 5S rRNA (Fig. 1G).

Although the ssl-sRNA based on the minimized scaffold is much shorter, we found ssl-sRNAs also require Hfq to exhibit its full repression activities as M7_anti-gfp sRNA only repressed gfp expression by ~40% in an E. coli BL21 (DE3) hfq-mutant (Fig. S2A). Scanning of the most
effective base pairing region and base pairing length of ssl-sRNAs was also conducted. It was found that 24 nt is the shortest base pairing (via perfect base pairing) length to confer full ssl-sRNA activity, and extension of the base pairing region did not obviously change the repression level (Fig. S2B). ssl-sRNAs pairing to the start codon and its ensuing 21 nt sequence conferred the highest repression activity, which is same to the property of synthetic sRNA with multiple stem-loop structures [15]. In contrast, ssl-sRNAs pairing to the 3′ UTR, 5′ UTR or to coding region rear to ATG start codon repressed weakly gfp expression (Fig. S2C). Moreover, we also tested the functionality of anti-gfp ssl-sRNA in B. subtilis and found that gfp expression was repressed by ~73% (Fig. S2D), suggesting the multispecies application potentials of ssl-sRNAs.

3.2. Ssl-sRNAs based on the minimized scaffold exhibit weak polar effect in regulating polysictron

In the studies of metabolic engineering or synthetic biology, gene repression is generally performed to chromosomal genes. To further prove the functionality of ssl-sRNA in regulating chromosomal gene, we attached the 24 nt base pairing region of chromosomal lacZ gene to generate M7_anti-lacZ ssl-sRNA, which repressed the expression of single-copy chromosomal β-galactosidase (lacZ) gene by 92.0% (Fig. 2A). This indicates chromosomal genes could be efficiently repressed by ssl-sRNAs.

Most prokaryotic genes are organized into polycistronic operons on the chromosomes. Gene repression via interference of transcription such as CRISPR interference, (CRISPRi) or destabilize the mRNA always suffer from severe polar effects and results in undesired downstream gene repression [36]. To explore the polar effect of ssl-sRNAs regulation, we constructed a polycistronic operon composed a gfp gene and BhepIII gene [24] (encoding the heparinases III of Bacteroides thetaiotaomicron) (Fig. 2) and found the expression of M7-anti-gfp sRNA repressed the expression of downstream BhepIII (Fig. 2C). Similarly, the expression of M7-anti-BhepIII ssl-sRNA repressed strongly BhepIII expression but did not affect the expression of upstream gfp gene (Fig. 2C). Taken together, these results suggest ssl-sRNA regulation unlikely causes polar effects and should be a more precise tool for identifying chromosomal engineering target genes in E. coli.

3.3. Resolve the core scaffold of ssl-sRNAs

On the basis of constructing the minimized M7 scaffold, we sought to find out whether other rho-independent terminators could also afford the construction of ssl-sRNAs. To this end, we used an artificial GadY sRNA [37], the classical conserved prokaryotic intrinsic terminators (CT) searched by Trans-TermHP [38], and the bacteriophage T7 terminator [39] (Fig. 3A) were trimmed to single stem-loop structures and applied to construct anti-gfp ssl-sRNAs (Fig. 3A). We found that the generated GadY_anti-gfp sRNA, T7_anti-gfp sRNA and CT_anti-gfp sRNA reduced GFP expression by 50.7%, 57.4% and 76.2% (Fig. 3A and Fig. S3). These three ssl-sRNAs were expressed at levels similar to those ssRNA (Fig. 3B). These results suggest that terminators with robust termina
tion activities (such as T7 terminator) are not necessarily good sRNA scaffolds activity (Fig. 3A). This might be ascribed to different stereo-hindrance effects when ssl-sRNA binds to mRNA or blocks ribosomes and different sRNA folding properties that determine the dynamic conformation of the ssl-sRNA structures.

We mutated GadY terminators stepwise by modifying the stem and loop lengths to explore the correlation between repression activities and terminator structures (Fig. 3C and D). Ten variants were created with variant 1 to variant 6 and variant 7 to variant 10 composed of progressively elongated (Fig. 2C) and shortened stems, respectively (Fig. 2D). We found that anti-gfp ssl-sRNAs constructed from variant 1, variant 2 and variant 3 exhibited the highest repression activities than other ssl-sRNAs (Fig. 3E and F). In particular, variant 3 anti-gfp ssl-sRNA of the GadY scaffold repressed gfp expression by 69.7% (Fig. 3E, in comparison to GadY Control sRNA). Taken together, ssl-sRNAs with stronger activities prefer the structure of a 7 bp stem, a 4 nt loop and a polyU (≥ 7 U) tail, which is defined as the core scaffold of ssl-sRNAs (Fig. 3G).

3.4. Automated design of ssl-sRNAs with programmable regulatory activities

It is evident that numerous nucleotide sequences can fold into the secondary structure of the core scaffold (Fig. 3G). We sought to explore whether core structures folded from different nucleotide sequences confer different ssl-sRNA activities. To this end, 17 additional core structures based on M7 and the GadY variant 3 (Fig. 3C) were created in four different ways (Fig. S4A) and applied to the construction of anti-gfp ssl-sRNAs. We found the newly created ssl-sRNAs displayed distinct repression activities (Fig. 4B).

Regression analysis was first performed to investigate the relationship between the minimum free energy of the core scaffolds by RNAfold [29] and the ssl-sRNAs activities. However, the correlation coefficient was not satisfying (R² = 0.64, Fig. 4A). Next, we explored the thermodynamic details of the core scaffolds and a set of their thermodynamic parameters were compiled by RNAeval [29]. Considering the polyU tail length of the terminator did not change the detailed thermodynamic description of the core structure, we decomposed the stem-loop structure of the core scaffold into six interior loops and a hairpin loop, and

Fig. 2. Repress chromosomal gene and polycistronic operon with ssl-sRNAs. (A) Repression of chromosomal β-galactosidase gene lacZ with M7_anti-lacZ sRNA. The expression of lacZ gene and M7_anti-lacZ sRNA was induced by IPTG. (B) and (C) Analysis the polar effect of ssl-sRNAs in regulating polycistronic operon. Genes gfp and BhepIII were assembled into an operon under the control of T7 promoter and terminator. Expression of gfp and BhepIII and the three ssl-sRNAs were induced with IPTG. Cells were collected after 12 h cultivation at 37 °C to measure the fluorescence intensity and enzymatic activities. SD, Shine–Dalgarno sequence; Fluorescence intensity/OD₆₀₀. Flu/OD₆₀₀. The data are expressed as the mean ± S.D. from three (n = 3, in C) or six (n = 6 in B) biologically independent replicates. Statistical evaluation (p value) was performed by two-sample t-test. **p < 0.01, *p < 0.05.
analyzed the thermodynamic description of each loop (Fig. 4B, Fig. S4B). No reliable linear relationship could be established between the thermodynamics of any single interior loop or the hairpin loop and the final ssl-sRNA activities. The thermodynamics of each interior loop appeared to contribute neither equally nor independently to the sRNA regulation activities (Figs. S4C–I). We concluded that the thermodynamic details and the distinct contribution factors ($\beta$) of the interior loops and hairpin loop to the anti-gfp ssl-sRNA activities should all be taken into consideration. Utilizing this set of physicochemical parameters of the core scaffolds and corresponding experimental GFP fluorescence data, a multiple linear regression model was developed to generate a scoring function for ssl-sRNA activity prediction and classification (Fig. 4B and Formula 1, $R^2 = 0.85$; Fig. S4B). Higher scores are correlated with higher ssl-sRNA repression activities, as indicated by lower gfp expression levels (Fig. 4B).

Fig. 3. Resolving the core scaffold for ssl-sRNAs design. (A) Creation of synthetic ssl-sRNAs from the trimmed GadY terminator, the trimmed conserved prokaryotic intrinsic terminator and the trimmed T7 terminator. The secondary structures of the terminators and the activities of GadY_anti-gfp sRNA, CT_anti-gfp sRNA and GadY_anti-gfp sRNA on repressing GFP expression. (B) Northern blot analysis of the expression of the anti-gfp ssl-sRNAs created in (A). 5S rRNA was used as the internal standard. Samples were cultivated for 6 h and collected to extract total RNA. Biotin-labeled probes binding to 5S rRNA or the 24 nt base pairing region of gfp were used to detect 5S rRNA or synthetic sRNAs. (C) and (D) Mutagenesis of the GadY terminator. The GadY terminator was progressively mutagenized by nucleotide substitutions, insertions or deletions in the stem–loop region to construct the designated mutants of the GadY terminator. (E) and (F) The activities of anti-gfp ssl sRNAs constructed from GadY mutants constructed in (C) and (D) on repressing GFP expression. All cultivations were performed at 37 °C in LB medium supplemented with 0.1 mM IPTG and necessary antibiotics. The expressions of plasmid-encoded gfp and ssl-sRNAs were driven by T7 RNA polymerase encoded in the genome of E. coli BL1 (DE3). Fluorescence intensity/OD$_{600}$, Flu/OD$_{600}$. Base pairing probabilities are indicated by the color gradient. All the data are expressed as the mean ± S.D. from three (n = 3) biologically independent replicates. (G) The core scaffold of ssl-sRNAs.
Fig. 4. Development of ssl-sRNAs scoring function and web-based de novo designer. (A) Correlation of ssl-sRNA activities and overall minimal free energy of the core scaffolds constructed in Fig. S4A. (B) A scoring function correlates the thermodynamic details of the decomposed secondary structures of the core scaffolds and the conferred anti-gfp ssl-sRNA activities analyzed by multiple linear regression (see Fig. S4B for calculation process). (C) Workflow of web tool for de novo design of ssl-sRNAs. A local Python program generates random sequences that would form core scaffolds upon the input of target sequences. NUPACK (4.0.0.23) is recruited to examine the overall structure of the generated ssl-sRNAs. Candidates with satisfactory secondary structures are transferred to RNAevel to analyze thermodynamic details. Afterward, the scores of the ssl-sRNAs are calculated to classify the sRNAs into groups with ‘strong repression’, ‘moderate repression’ and ‘weak repression’. (D) Snapshots of the interfaces of the input and output interfaces of the web designer. (E) Workflow of ssl-sRNA expression vector construction. Customer-defined promoters and ssl-sRNA sequences are all automatically included in sslRNAD design primers (two pairs) that can be used for PCR experiments with desired plasmids. The PCR fragments become self-cyclized in E. coli cells via the homologous ends conferred by the primers to form the ssl-sRNA expression vectors.
3.5. Control E. coli cell morphology with anti-ftsZ ssl-sRNA

Next, we designed an ssl-sRNA with strong anti-ftsZ activity to regulate cell morphology. FtsZ is the conserved protein that forms the septal ring structure during cell division [41]. Depletion of FtsZ leads to the formation of filamentous cells [42]. As the FtsZ protein is crucial for E. coli, it is difficult to create ftsZ deletion mutants by editing the genome. We demonstrated that by transforming a recombinant plasmid carrying the constitutively expressed anti-ftsZ ssl-sRNA, the rod-shaped E. coli cell morphology was easily transformed into a filamentous morphology (Fig. 5). Expression of the anti-ftsZ ssl-sRNA by the Anderson J23105 promoter with weaker promoter strength changed the E. coli cell morphology to a slightly filamentous morphology (Fig. 5), while upon increasing the expression of anti-ftsZ ssl-sRNA with a stronger Anderson J23100 promoter (promoter strength: approximately 3-5-fold of J23105), the cells became significantly longer (Fig. 5). Fluorescence microscopy after Nile red staining also demonstrated that filamentous cells were not dividing normally because the downregulation of the ftsZ gene disrupted the normal membrane fission process (Fig. 5).

3.6. Identify new metabolic engineering targets of ergothioneine biosynthesis with an ssl-sRNA library

Ergothioneine is a sulfur-containing histidine derivative synthesized by many fungi and some bacteria. The superantioxidant nature of ergothioneine has made it an invaluable ingredient in foods [43] and cosmetics [44]. Several genes have been engineered for improving ergothioneine biosynthesis in industrial microorganisms such as E. coli [45] and Saccharomyces cerevisiae [46]. But synthesis of ergothioneine is a very complicated process involving histidine, methionine, cysteine and glutamate and S-adenosyl methionine (SAM) [44] as precursors (Fig. 6A).

To identify new target genes for enhancing the biosynthesis of ergothioneine, we selected 80 gene candidates including pathway genes, transporter genes and regulator genes that may control the availability of precursors, SAM, cysteine, methionine, glutamate and histidine (Fig. 6A and Table S3), and developed a workflow for rapidly creating the ssl-sRNA library (Fig. 6B). After performing one-pot PCR with all the primers (for targeting the 80 genes) designed by ssl-sRNA in one tube, the PCR produces carrying the ssl-sRNA library were purified and transformed directly into E. coli BL21 (DE3)/pRSF-egfBCDE (the ergothioneine production parental strain) as depicted in Fig. 4E. Via Amplicon Sequencing (by GENEWIZ, Suzhou, China), we confirmed ssl-sRNAs targeting 79 out of the 80 selected genes were created successfully by the one-step PCR method (Table S4), indicating ssl-sRNA library was composed diversified ssl-sRNAs.

Transformants of the PCR product (213 colonies, picked randomly on the LB plate) and colonies of the parental strain carrying M7_Control ssl-sRNA expression vector (3 colonies, used as the control strains) were inoculated into nine 24-well plates. Ergothioneine produced by the transformants and the control strain were measured directly by HPLC prior to figuring out which gene is repressed in every transformant (Fig. 6C). After 32 h cultivation in 24 well plates, it was found 15 transformants produced higher level of ergothioneine and 11 transformants produced much lower level of ergothioneine than the control strain (Table S5). Moreover, we detected 13 different ssl-sRNAs in the 15 transformants produced higher level of ergothioneine and 11 transformants produced much lower level of ergothioneine than the control strain (Table S6), with the other two transformants uncharacterized because of impurity of transformant of 9D-5 and unknown DNA insert in the vector isolated from transformant of 9D-6 (Table S6). Minor mutations were found in the 13 characterized ssl-sRNAs, but the overall structure and base pairing capabilities of the ssl-sRNAs are unlikely affected (Table S6 and Table S7). Therefore, we applied the 13 transformants with increased ergothioneine productivities to shake flask cultivations and confirmed 8 of them indeed produced higher level of ergothioneine than the control strain (Fig. 6D). The identified target
genes are covering the central carbon metabolism pathway (ppc, pck), the consumption of cysteine and methionine (cysS, metG), nitrogen metabolism regulation (glnE, nac), the biosynthesis of UMP/Pyrimidine (pyrF, pyrE), the biosynthesis of serine (tdcB, sdaA). This once again highlights the complexity of microbial metabolism regulation and the necessities of developing new gene regulation tools, such as sRNA.

Taken together, these validation experiments demonstrated that the web-based sslRNAD platform can rapidly create a series of customized ssl-sRNAs or ssl-sRNA libraries, which could be easily expressed from one-pot PCR-constructed vectors and applied to the fields of synthetic biology and metabolic engineering.

4. Discussion

sRNAs created for the repression of non-cognate genes are mainly constructed by replacing the base pairing region of natural sRNAs. Most natural sRNAs fold into complicated secondary structures which makes the design of artificial sRNAs rather challenging. Due to limited natural sRNA scaffolds, the activities of synthetic sRNA are not programmable. In this study, we found that rho-independent terminators with the structure of a 7 bp stem, a 4 nt loop and a polyU (7 or longer) tail afforded the construction of single stem-loop sRNAs (ssl-sRNAs) with secondary structures reduced to essentials (Figs. 1–3). We have developed a new sRNA sequence-structure-activity model, and created an easy-to-use web tool for de novo design of ssl-sRNAs (Fig. 4). ssl-sRNAs are less likely to fold into unwanted secondary structure because the server filtered incorrectly folded candidates (Fig. 4). The activities of ssl-sRNAs are highly programmable. Moreover, as the ssl-sRNAs are very short in length, their DNA sequences as well as the interfaced promoters can be incorporated into overlapping primers, which are designed simultaneously by the web server and applied to PCR with designated plasmids as templates. The PCR products are automatically developed into ssl-sRNA expression vectors after transformation into E. coli competent cells.

The interaction of Hfq with sRNA relies heavily on the polyU tail at the 3’ terminus [8,32]. Therefore, all our designed ssl-sRNAs essentially have a polyU tail. A previous study also suggested the involvement of the ‘AU’ region in the regulatory activity of SgrS sRNA toward its cognate target, pssG mRNA and the binding of Hfq [32]. In contrast, our study demonstrated that mutation or deletion of this ‘AU’ region slightly altered the ssl-sRNA activities (Fig. 1). In addition to binding the ‘AU’ sequences, a recent crystal structure study on the Hfq-dsDNA complex suggested that the Hfq protein should also bind the double-stranded stems of rho-dependent terminators in a sequence-independent manner [47], which provides structural mechanism for the regulatory function of single ssl-sRNAs designed in this study.

Although the rho-independent terminators could all potentially be applied to ssl-sRNA design, our results demonstrated that some terminators conferred apparently stronger sRNA activities than the other terminators (Fig. 3). We termed the most competent scaffold with
elements of a 7 bp stem, a 4 nt loop and a polyU tail the core scaffold for sRNA design (Fig. 3).

Dynamic control of gene expression is always required in synthetic biology and metabolic engineering studies. ssl-sRNAs designed with different activities could be easily interfaced synergistically with constitutive and inducible promoters to achieve more tunable regulation performance (Fig. 5) because gene regulation activities are determined not only by sRNAs themselves but also by the concentrations of the target mRNA or Hfq protein [13, 48, 49].

When screening large gene sets, ssl-sRNA library with strong activity could be first designed and experimentally constructed following the streamlined workflows (Figs. 4E and 6B). Additionally, we found that M7 (Fig. 1F) can also function as a scaffold for creating active ssl-sRNAs for the model Gram-positive bacterium Bacillus subtilis (Fig. S2D), suggesting the potential applications of sslRNAD across different chassis microbes.

Data availability
All data needed to evaluate the conclusions in the paper are present in the paper and/or Supplementary Data. sslRNAD is available for academic users and is provided as a web server which can be accessed with the following link: http://www.kangzlab.cn/. The source code of sslRNAD is publicly available on Zenodo (http://doi.org/10.5281/zenodo.6914471). The Amplicon sequencing results of sRNA is available at Sequence Read Archive (SRA) of NCBI, PRJNA893451. http://www.ncbi.nlm.nih.gov/bioproject/PRJNA893451. Other information is available from the corresponding author on request.

CRediT authorship contribution statement
Yang Wang: Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review & editing. Guobin Yin: Software, Investigation. Huanjiao Weng: Investigation. Luyao Zhang: Investigation. Guocheng Du: Project administration. Jian Chen: Supervision. Zhen Kang: Funding acquisition, Conceptualization, Writing
Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2022.11.006.

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