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Development of multiplexing gene silencing system using conditionally induced polycistronic synthetic antisense RNAs in *Escherichia coli*

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ABSTRACT

Although efficient methods of gene silencing have been established in eukaryotes, many different techniques are still used in bacteria due to the lack of a standardized tool. Here, we developed a convenient and efficient method to downregulate the expression of a specific gene using ~140 nucleotide RNA with a 24-nucleotide antisense region from an arabinose-inducible expression plasmid by taking *Escherichia coli lacZ* and *phoA* genes encoding β-galactosidase and alkaline phosphatase, respectively, as target genes to evaluate the model. We examined the antisense RNA (asRNA) design, including targeting position, uORF stability elements at the 5′-end, and Hfq-binding module at the 3′-end, and inducer amount required to obtain effective experimental conditions for gene silencing. Furthermore, we constructed multiplexed dual-acting asRNA genes in the plasmid, which were transcribed as polycistronic RNA and were able to knockdown multiple target genes simultaneously. We observed the highest inhibition level of 98.6% when *lacZ* was targeted using the pMKN104 asRNA expression plasmid, containing a five times stronger P_{BAD} -10 promoter sequence with no requirement of the Hfq protein for repression. These features allow the system to be utilized as an asRNA expression platform in many bacteria, besides *E. coli*, for gene regulation.

1. Introduction

Synthetic asRNA-mediated gene silencing technology has been widely used as a conventional tool. The small RNAs (sRNAs) in eukaryotes, such as siRNA and miRNA, which are typically 18–25 nt in length, have become important tools to specifically knock down gene expression in various target cells [1]. However, prokaryotes do not have a bioprocess mechanism for RNAi, thus it is expected that versatile silencing technology will be established, with many researchers testing various gene silencing methods [2,3]. In bacteria, various types of customized synthetic asRNA expressed from a plasmid are typically designed to inhibit translation by base-pairing with target mRNAs [4–6]. Further, most gene silencing by asRNAs in metabolic engineering is required for the RNA chaperon protein Hfq and the binding sequences at the termination position of the asRNA-containing transcript [7,8]. The Hfq protein stimulates RNA-RNA binding and degradation of the target mRNA [9]. The synthetic asRNA gene knockout system developed in *E. coli* was also applied to other bacteria, such as *Clostridium acetobutylicum* [10] and *Corynebacterium glutamicum* [11]. However, *E. coli* Hfq introduction into the strains was required for efficient gene silencing, since no functional Hfq homologues have been found in these bacteria [10].

The plasmid-based expression of synthetic asRNAs allows convenient genome-scale gene regulation of multiple targets compared to the time-consuming conventional genome engineering method [12]. Multiple gene silencing technology is especially useful for metabolic engineering and gene network study because multiple mRNAs can be targeted by independently induced asRNAs. Despite these advantages, there are several experimental limitations, such as the use of compatible plasmids, multiple antibiotic-resistance genes and antibiotics, and the consideration of

Abbreviations: sRNA, small RNA; asRNA, antisense RNA; IPTG, isopropyl-β-D-thiogalactopyranoside; SD, Shine-Dalgarno sequence.

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promoters that utilize different repressors and inducers [8,13]. In addition, multiple transformations are required for combining plasmids in a cell, which is time-consuming and labor-intensive. Therefore, we developed a novel method that introduced multiple asRNA gene cassettes into one type of plasmid and transcribed them from a single promoter by adding an inducer to inhibit the expression of multiple genes simultaneously, enabling reduction of the number of plasmids and rapid and efficient modulation of target gene expression.

In the present study, we describe the systematic analysis of asRNA-based gene inhibition tools using endogenously expressed *lacZ* and *phoA* genes. This work developed a single plasmid-based inducible asRNA expression platform for multiple gene silencing, which does not require Hfq protein and the scaffold sequence. Thus, this tool can be utilized in a variety of bacteria used in the industrial production of various biochemicals. This is the first study to show multiplexing gene silencing of chromosomally encoded genes using conditionally induced polycistronic synthetic asRNAs.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Supplementary Table S1. The *E. coli* DH5α strain was used as the cloning host and grown in Luria-Bertani broth (LB) medium containing chloramphenicol (Cm; 25 μg/mL) at 37 °C with continuous shaking at 200 rpm, with or without 0.2% L-arabinose, or on LB agar plates containing 1.5% (wt/vol) agar supplemented with Cm (25 μg/mL) at 37 °C with or without 0.2% L-arabinose.

2.2. Construction of synthetic asRNA expression plasmids

The oligonucleotides and primers used in this study are listed in Supplementary Table S2. To generate pMKN101 plasmid, in which no insert sequence was cloned between *Xba*I and *Spe*I sites, such that the standard BioBrick prefix and suffix sequences [14] could be used for the cloning of any nucleotide sequence to express a transcript under the P_{BAD} promoter, the BioBrick sequence was amplified via PCR using primers EcoNotXbaI-F1 and SpeXbaNot-R1, and cloned into the *Eco*RI and *Spe*I sites of pBBtt-ldrDx [15]. To generate pMKN104 plasmid, which increases expression from the P_{BAD} promoter, the −10 box sequence (TACTGTT) was changed to the −10 box sequence of *lacUV5* (TATAATG) using the PrimeSTAR Mutagenesis Basal Kit (Takara Bio, Kusatsu, Shiga, Japan). Nucleotide sequence was confirmed using DNA sequencing (Fasmac, Atsugi, Kanagawa, Japan).

2.3. Preparation of synthetic asRNA insert

PCR was performed using a PrimeSTAR Max DNA Polymerase (Takara Bio) and forward (F) and reverse (R) primers that contained restriction sites and partially complimentary sequences (asRNA sequences) to prepare insert DNA fragments with a 24-nt antisense sequence. The PCR product was treated with *Xba*I and *Spe*I, then column purified using NucleoSpin Gel and a PCR Clean-up procedure (Takara Bio). The insert DNA was ligated into the *Xba*I and *Spe*I sites of pMKN101 using a Mighty Mix DNA Ligation Kit (Takara Bio). To prepare multiple asRNA inserts, an upstream insert DNA fragment was treated with *Eco*RI and *Xba*I, and a downstream insert DNA fragment was treated with *Spe*I and *Pst*I. Those insert DNAs were ligated into the *Eco*RI and *Pst*I sites of pMKN101. Ligation mixtures were transformed into DH5α competent cells by electroporation and plated on LB agar plates containing Cm (25 μg/mL). Plasmids were sequenced using Fasmac (Atsugi, Kanagawa, Japan).

2.4. β-galactosidase assay

MG1655 P_{CP18}-*araE* harboring a parent plasmid (pMKN101 or pMKN104) or the asRNA insert containing plasmids were grown at 37 °C overnight in LB medium containing Cm (25 μg/mL), then diluted (1:200) into fresh LB medium containing Cm (25 μg/mL), IPTG (1 mM), and L-arabinose (0.2%). At mid log phase (OD_{600} of 0.6), the culture was divided in three and incubated on ice. Cultures (100 μl) (in triplicate), 20 μl chloroform, 10 μl 0.1% (W/V) SDS, and 900 μl Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7.0) were mixed at 28 °C. Optical density was measured at OD_{600} using a Shimadzu UV-1800 UV–Vis spectrophotometer (Shimadzu Corp., Kyoto, Japan). The reaction was started with 200 μl ONPG (4 mg/ml) at 28 °C and stopped with 500 μl 1 M Na_2CO_3, and cells were collected using centrifugation at 10,000 × g for 3 min at room temperature. Thereafter, the supernatants were transferred into a cuvette. Each 1 ml aliquot was measured at OD_{420} and OD_{550} using a spectrophotometer.

2.5. Alkaline phosphatase assay

Screened constitutive expressing *phoA* mutant (MG1655 P_{CP18}-*araE phoA* mt1) harboring a parent plasmid (pMKN101 or pMKN104) or the asRNA insert containing plasmids were grown at 37 °C overnight in LB medium containing Cm (25 μg/mL), then diluted (1:200) into fresh LB medium containing Cm (25 μg/mL) and L-arabinose (0.2%). At mid log phase (OD_{600} of 0.6), the culture was collected and incubated on ice. Cells were collected from 1 ml culture and pellets were resuspended with 1 ml resuspension buffer (10 mM Tris-HCl, 0.1 M NaCl) and transferred into a cuvette. Optical density was measured at OD_{600} using the spectrophotometer. Each 100 μl aliquot (in triplicate) was incubated on ice. Then, 900 μl Tris-HCl, pH 8.0, 60 μl chloroform, and 30 μl 0.1% (W/V) SDS were mixed and incubated for 5 min at 37 °C. The reaction was started with 100 μl PNPP (4 mg/ml) at 37 °C, stopped with 100 μl 1 M K_2HPO_4, and incubated on ice. Cells were collected using centrifugation at 10,000 × g for 3 min at 4°C, then supernatants were transferred into a cuvette. Each 1 ml aliquot was measured at OD_{420} and OD_{550} using a spectrophotometer.

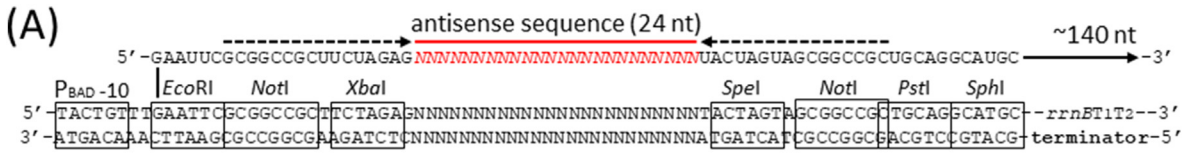
2.6. Northern blot analysis

Total RNA was collected from the DH5α strain harboring a parent plasmid (pMKN101 or pMKN104) 1 h after adding distilled water (−) or 0.2% L-arabinose (+) at OD_{600} ~0.3. rRNA (5S, 18S, and 23S) bands were visualized using methylene blue staining. Total RNA (10 μg) was loaded into each lane of a 1% (w/v) agarose-formaldehyde gel. After electrophoresis, RNA was blotted on a positively charged nylon membrane using a Whatman Nytran SuPerCharge TurboBlotter kit (Merck KGaA, Darmstadt, Germany) and hybridized using 100 pmol digoxigenin (DIG)-labelled DNA oligo probe (GeneDesign, Ibaraki, Osaka, Japan) at 50 °C overnight. The ApBBtf-DIG-1 probe is complementary to the *Sph*I-*Pst*I-*Not*I-*Spe*I region in the synthetic insert transcript (Fig. 1A). Hybridization and detection protocols were provided by Roche Diagnostics (Basel, Switzerland). The signal was visualized using a LAS-3000 mini-image analyzer (FujiFilm, Tokyo, Japan).

3. Results

3.1. Design and construction of inducible synthetic asRNA expression plasmids

To knockdown any target gene of interest in *E. coli*, we first generated a synthetic asRNA expressing plasmid, pMKN101



(B)

lacZ mRNA 5′-AACAAUUUCACACAGGAAACAGCUAUGACCAUGAUUACGGAUUCACUGGCC-3′

ASlacZD: 3′-UACUGGUACUAAUGCCUAAGUGAC-5′

ASlacZM: 3′-UCCUUUGUCGAUACUGGUACUAAU-5′

ASlacZU: 3′-UUAAAGUGUGUCCUUUGUCGAUAC-5′

(C)

phoA mRNA 5′-GUAUUUGUACAUGGAGAAAAUAAAGUGAAACAAAGCACUAUUGCACUGGCA-3′

ASphoAD: 3′-CACUUUGUUUCGUGAUAACGUGAC-5′

ASphoAM: 3′-CUCUUUUAUUUCACUUUGUUUCGU-5′

ASphoAU: 3′-AAACAUGUACCUCUUUUAUUUCAC-5′

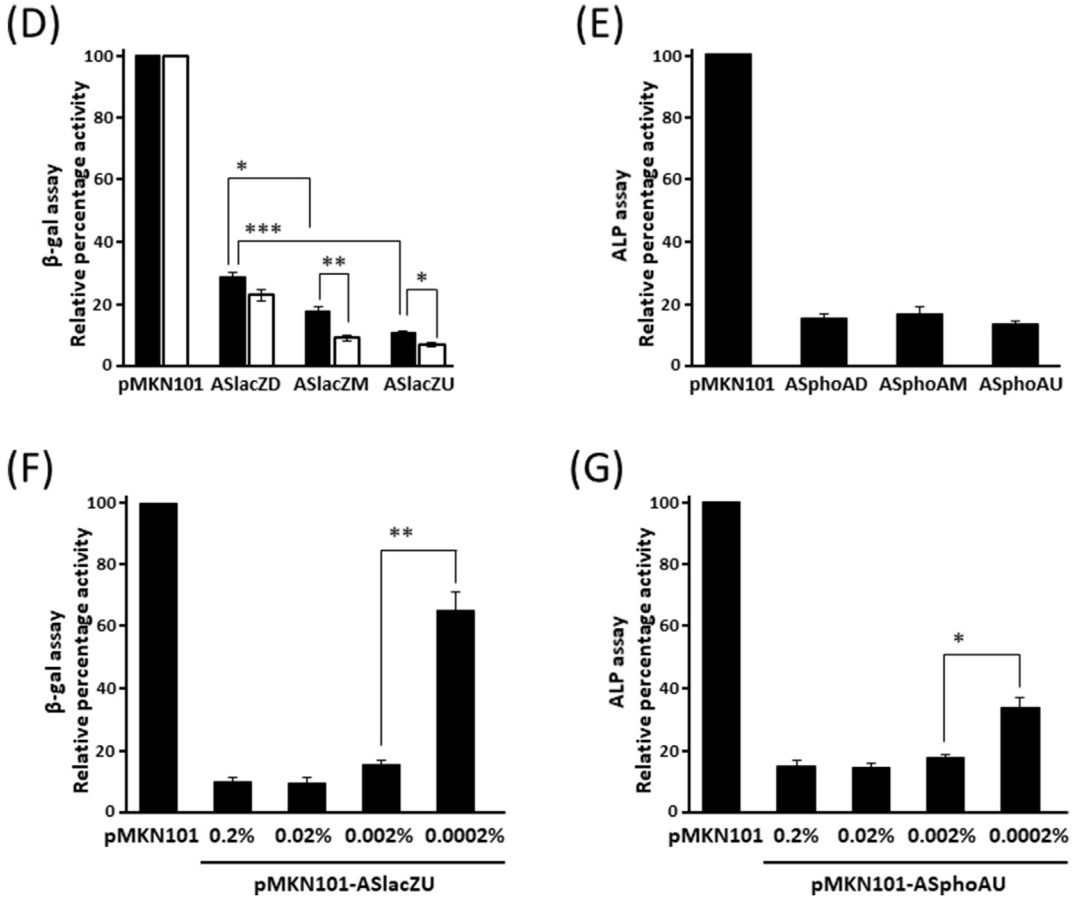


Fig. 1. Plasmid-based synthetic small asRNA design. (A) Transcription region of the pMKN101. Promoter and restriction enzyme recognition sites are boxed. Transcription initiation and termination sites are indicated by a black folding arrow from G at the *Eco*RI site. Red *N*s show 24-nt asRNA sequence complementary to the translation initiation region of target mRNAs. Inverted dotted arrows and the red bar indicate the potential stem-loop structure region in the transcript. (B and C) Organization of synthetic asRNAs. The antisense sequence (24 nt; italic and red) is complementary to the downstream (shown as D), middle (shown as M), or upstream (shown as U) section of the translation initiation region of target mRNAs. Sequences of the translation initiation region of *lacZ* (B) and *phoA* (C) mRNAs (upper). SD sequences and translation initiation codons are underlined. Enzymatic activities of β-galactosidase (*lacZ*) and alkaline phosphatase (*phoA*) were measured in cell extracts of MG1655 P_{CP18}-*araE* with 1 mM IPTG and MG1655 P_{CP18}-*araE phoA* mt1,

(Fig. 1A), which was used as a control plasmid throughout the experiments. The sRNA expression plasmid pMKN101 expressed an approximately 120-nt sRNA, when no insert was included, including *rrnB*T_1T_2 transcription terminator sequences at the 3′ end under the control of the P_{BAD} promoter [16]. We designed a 24-nt antisense sequence that was complementary to the translation initiation region of the target mRNA. A 24-nt sequence is long enough to ensure high affinity to target *E. coli* transcripts and short enough to avoid cross-reaction, since a longer target-binding sequence of synthetic asRNA may cause cross-reactivity [5].

To determine knockdown efficiency of our asRNA gene silencing system, we chose the *lacZ* and *phoA* genes as targets because those expression levels were easily and accurately determined by enzymatic activity tests (β-galactosidase and alkaline phosphatase activity, respectively) in *E. coli*. We first constructed three types of asRNA expressing plasmids to repress gene expression of *lacZ* or *phoA* (Fig. 1B and C, and Supplementary Table S1). The first plasmid was designed to produce asRNAs that can base-pair with the start codon and the following downstream 21-nt coding sequences (pMKN101-ASlacZD and pMKN101-ASphoAD). The second plasmids were designed to produce asRNAs that bind to the sequence that spans the SD sequence to nucleotide +13 of the *lacZ* (pMKN101-ASlacZM) and *phoA* mRNA (pMKN101-ASlacZM). The third plasmids express asRNA complementary to the translation signal region, including the SD sequence and start codon of the mRNA target (pMKN101-ASlacZU and pMKN101-ASphoAU).

3.2. Gene knockdown by asRNAs targeting around the initiation codon

To examine whether synthetic asRNAs regulated endogenous gene expression, wild-type *E. coli* MG1655 with the P_{CP18}-*araE* promoter, which allowed homogeneous expression from P_{BAD} at a given inducer concentration (Supplementary Table S1) [17], was transformed with the series of the pMKN101-based asRNA expression plasmids. Initially, the β-galactosidase activity was determined in *E. coli* harboring pMKN101-ASlacZD, pMKN101-ASlacZM, pMKN101-ASlacZU, or the parent plasmid pMKN101. In order to induce the chromosomal *lac* operon, IPTG was added to the cultures. Upon induction with only IPTG or with IPTG and arabinose, the β-galactosidase activity was determined using a β-galactosidase assay. In the presence of IPTG alone, no significant differences were detected between extracts isolated from *E. coli* harboring pMKN101-ASlacZD, pMKN101-ASlacZM, pMKN101-ASlacZU (Fig. 1B), or the parent plasmid pMKN101 (data not shown). On the contrary, when both IPTG and arabinose were added to the culture medium, cells containing pMKN101-ASlacZD, pMKN101-ASlacZM, or pMKN101-ASlacZU showed decreased β-galactosidase activity when compared with cells harboring pMKN101 (black bar in Fig. 1D). Cells expressing ASlacZU asRNA showed a stronger repression of β-galactosidase activity, indicating a reduction to 10.5% (89.5% inhibition) compared to that of control cells containing pMKN101 plasmid.

Next, we examined whether asRNA gene silencing was Hfq dependent, although our asRNA transcript had no Hfq binding sequence. Interestingly, all *E. coli* Δ*hfq* mutant cells expressing asRNA targeting *lacZ* showed significant gene repression (White bar in Fig. 1D). The cells expressing ASlacZU showed the strongest repression of β-galactosidase activity between three asRNAs targeting *lacZ*, showing a reduction to 6.5% (93.5% inhibition) compared to the level in pMKN101/Δ*hfq* cells. Therefore, gene silencing by the asRNA was not Hfq-dependent, and Hfq may not stabilize the duplexes between asRNA and its target mRNA for gene silencing by our system because the sRNA, including the asRNA region transcribed from pMKN101, did not contain the scaffold sequence containing an Hfq-binding motif. It has been reported that Hfq decreased the gene silencing by a *cis*-acting type of asRNA that did not contain Hfq-binding sequences [6].

To further evaluate our asRNA silencing system, we examined a series of asRNAs targeting alkaline phosphatase protein coding genes *phoA*. Alkaline phosphatase activity was determined in cultures of *E. coli phoA* constitutive expression mutant (MG1655 P_{CP18}-*araE phoA* mt1) harboring pMKN101-ASphoAD, pMKN101-ASphoAM, pMKN101-ASphoAU (Fig. 1C), or the parent plasmid pMKN101. A similar trend was observed in the alkaline phosphatase assay. The cells expressing ASphoAU targeting the start codon and the upstream region, including the SD sequence, showed a stronger repression of alkaline phosphatase activity of 12.8% (87.2% inhibition) of the level observed in control cells containing the pMKN101 plasmid. We found that upstream asRNAs (ASlacZU and ASphoAU) were most effective in knocking down target genes. Therefore, we employed the upstream asRNA design for subsequent experiments.

3.3. Gene silencing effects of varying concentrations of arabinose

In order to better evaluate the effect of arabinose on asRNA expression, the LacZ and PhoA activities were determined as a function of the arabinose concentration in the culture medium using MG1655 P_{CP18}-*araE* harboring pMKN101-ASlacZU, or MG1655 P_{CP18}-*araE phoA* mt1 harboring pMKN101-ASphoAU. We observed decreased repression levels at arabinose concentrations of 0.002% and 0.0002%, while repression levels plateaued when the arabinose concentration ranged from 0.02% to 0.2% by both asRNAs (Fig. 1F and G). This result agrees with a previous report indicating that arabinose concentration at 0.02% and 0.2% rendered the highest expression from the P_{BAD} promoter, but showed a slightly reduced expression level at 2% concentration [17].

3.4. Effects of uORF and/or Hfq-binding motif on gene silencing

We further investigated the effect of RNA stabilization and Hfq-binding sequences for repression by asRNAs. It is shown that the presence of the intact SD sequence followed by the short ORF at the 5′ region of the asRNAs affects RNA stability and silencing effectiveness [18]. To increase the cellular stability of the tested asRNAs, the stabilizer element (SD sequence followed by the short ORF sequence: uORF) [19] was inserted at 5′-ends of the asRNA. The most compact Hfq-binding module (SgrSΔ14), which helps facilitate base-pairing between asRNAs and target mRNAs [20], was inserted at 3′-ends of the asRNA. We created ASlacZU and ASphoAU, which combines one or two these modules, to observe if they

respectively (black bar), or the corresponding *hfq* knockout strain (white bar), induced by 0.2% L-arabinose, expressing asRNAs targeting *lacZ* (D) and *phoA* (E), respectively. Enzymatic activities measured in cells expressing ~120-nt transcript without asRNA sequences from pMKN101 control plasmids were considered 100%. Knockdown efficiencies were measured by relative enzymatic activities. Asterisks indicate significant differences between different strains tested using a two-sample *t*-test (*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$).

Gene repression activities under different induction conditions. Enzymatic activities of β-galactosidase and alkaline phosphatase were measured in MG1655 P_{CP18}-*araE* with 1 mM IPTG and MG1655 P_{CP18}-*araE phoA* mt1 extracts, respectively, induced by 0.2%, 0.02%, 0.002%, or 0.0002% L-arabinose, expressing asRNAs (ASlacZU and ASphoAU) targeting *lacZ* (F) and *phoA* (G), respectively. All values represent mean ± standard deviation (s.d.) (n = 3). Each graph is representative of at least three independent experiments. (*$p < 0.05$, **$p < 0.005$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

improve gene silencing efficiency (Fig. 2A). Thereafter, β-galactosidase and alkaline phosphatase activity were determined using cells expressing one of those asRNA sequences, respectively. Results indicate that the addition of those elements did not obviously increased gene repression efficiency when compared to asRNA without elements (Fig. 2B and C). Thus, it is expected that the flanking transcript expressed from P_{BAD} on pMKN101 already contains nucleotide sequences enough to stabilize the asRNA. It may consist of a 16-nt stem structure (shown by inverted dotted arrows in Fig. 1A) adjacent to an asRNA insert and contains *rrnB*T_1T_2 rho-independent transcription terminator at the 3′-ends of the arabinose-inducible transcript (Fig. 1A).

3.5. Gene inhibition by dual-acting asRNAs

Next, to examine the usability of the synthetic asRNA system in multiplex gene silencing, *lacZ* and *phoA* were repressed simultaneously by expressing a single transcript with multiple asRNA binding sequences. The pMKN101 plasmid has a BioBrick assembly sequence as the site for inserting the asRNA insert sequence (Fig. 1A), which allows for the easy addition of the insert DNA sequence cassette. Antisense (AS) insert digested with *Xba*I or *Spe*I retains the same overhang sequence, allowing the two fragments to be ligated. Moreover, since the concatenated sequence is a scar sequence that is not cleaved by *Xba*I or *Spe*I, the *Xba*I and *Spe*I sequences can be used again after this fragment is concatenated to the *Eco*RI and *Pst*I sites of the pMKN101 (Fig. 3A). In this construct, both *lacZ* and *phoA* genes were targeted simultaneously and the same cell extracts were used for measurement of β-galactosidase and alkaline phosphatase activity to evaluate gene repression efficiency. Both ASlacZU + ASphoAU and ASphoAU + ASlacZU double asRNA insert expression strains induced significant repression of control enzymatic activity with ~86% inhibition of β-galactosidase activity (Fig. 3B) and 67–74% inhibition of alkaline phosphatase activity (Fig. 3C). Although those dual target asRNAs exerted lower gene silencing effects than single asRNAs, including ASlacZU or ASphoAU asRNA, which caused more than 87% inhibition, these results suggest the potential of the synthetic asRNA system in regulating multiple genes. On the contrary, double asRNAs, including dual inserts targeting the same gene, showed slightly higher gene silencing effects, with 94.2% inhibition (ASlacZU + ASlacZU), than single unit ASlacZU (~90% inhibition), but the ASphoAU + ASphoAU tandem *phoA* asRNA (83.1% inhibition) exerted similar gene silencing effects to individual gene knockdown of ASphoAU (~87% inhibition). In addition, asRNA targeting *phoA* mRNA (ASphoAU + ASphoAU) did not reduce β-galactosidase activity, and asRNA targeting *lacZ* mRNA (ASlacZU + ASlacZU) did not reduce alkaline phosphatase activity. These results indicate that the observed *lacZ* and *phoA* silencing triggered by those corresponding asRNAs is specific to the target gene.

3.6. Enhancing gene repression by asRNA with increased transcription

We further examined whether the synthetic asRNA system can knock down genes more efficiently by increasing asRNA transcript from the P_{BAD} promoter. We attempted to introduce mutations to increase expression from the P_{BAD} promoter, the −10 box sequence (TACTGT) was changed to the −10 box sequence of *lacUV5*

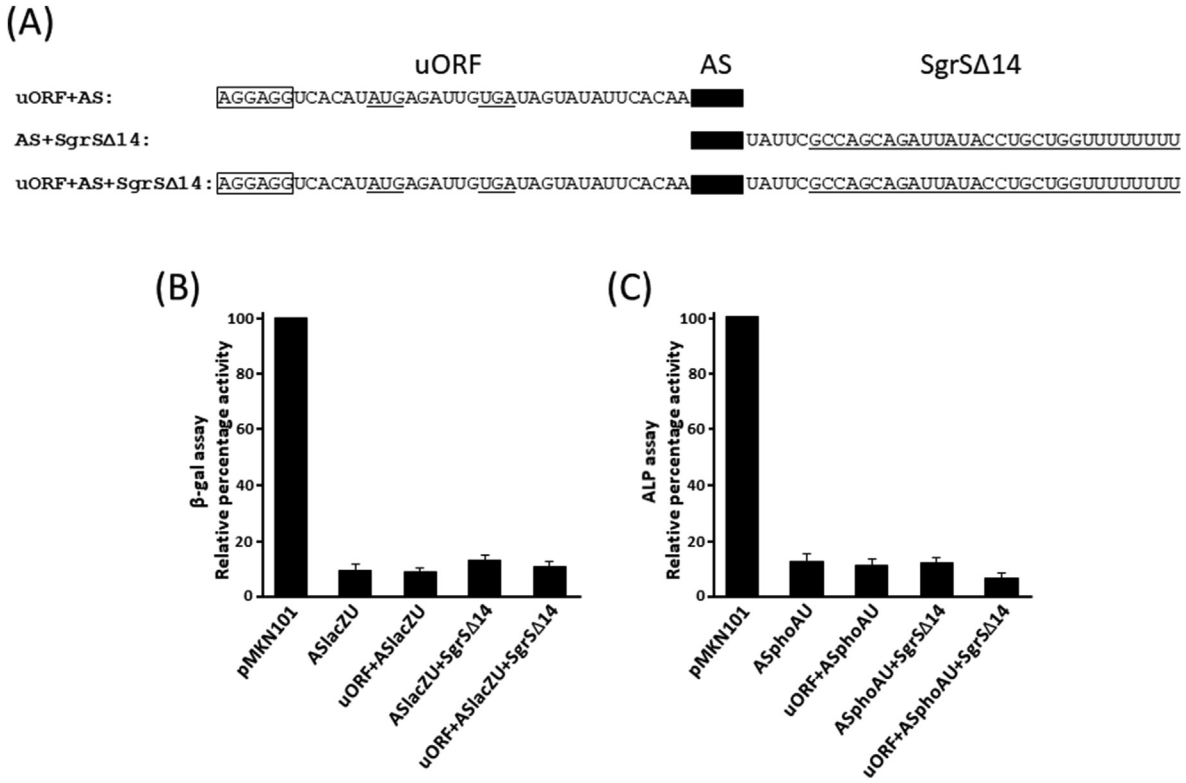


Fig. 2. Combined synthetic asRNA expression system. (A) Schematic representation of the customized asRNAs. The upstream ORF sequences (uORF) were fused to 5′-ends of asRNA sequences shown in black bar (uORF + AS). SD sequences are boxed. Translation initiation codons and stop codons are underlined. The Hfq-binding module (SgrSΔ14) was fused to 3′-ends of asRNA sequences (AS + SgrSΔ14). Enzymatic activities of β-galactosidase and alkaline phosphatase were measured in MG1655 P_{CP18}-*araE* with 1 mM IPTG and MG1655 P_{CP18}-*araE phoA* mt1 cell extracts, respectively, induced by 0.2% L-arabinose, expressing asRNAs targeting *lacZ* (B) and *phoA* (C), respectively. All values represent mean ± s.d. (n = 3). Each graph represents at least three independent experiments.

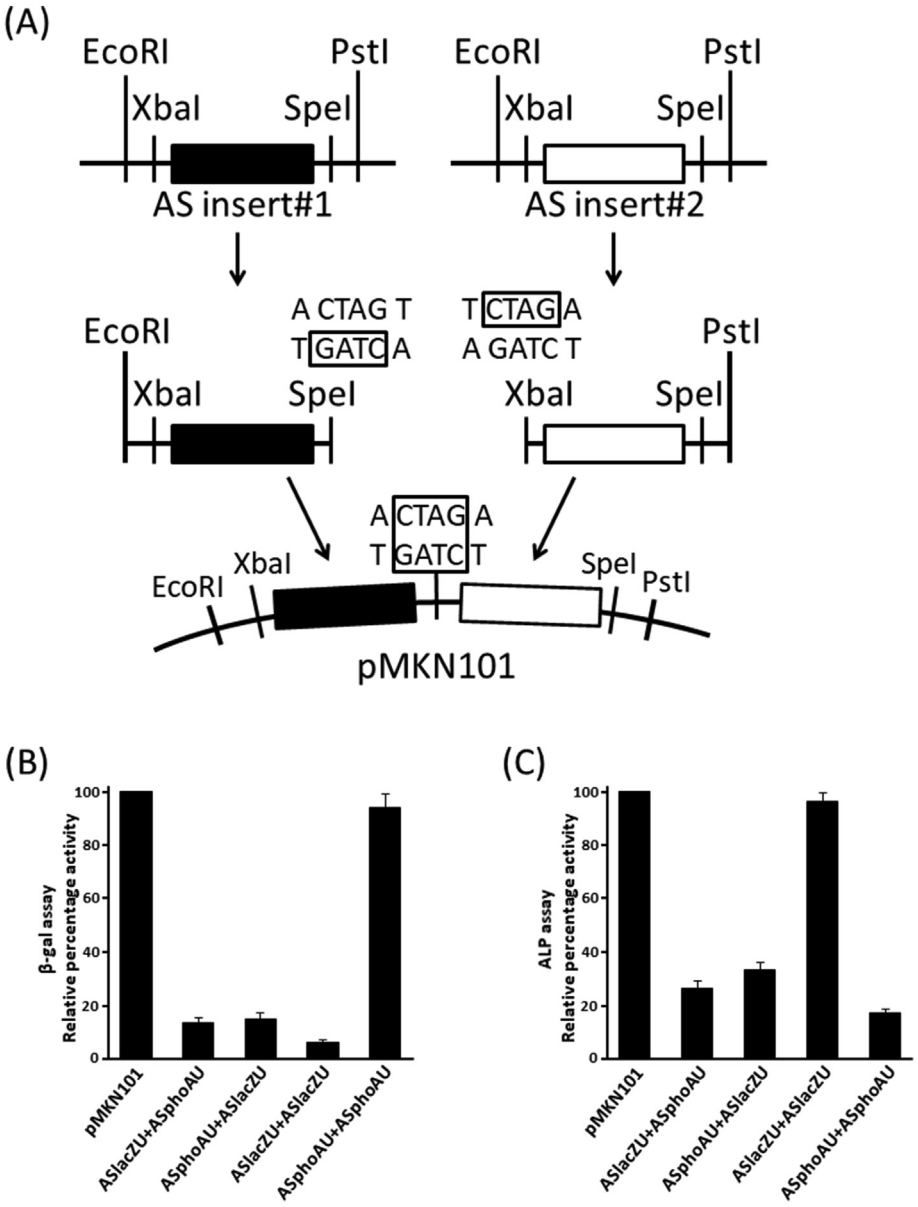


Fig. 3. Tandemly arranged synthetic asRNA expression system. (A) Schematic diagram of assemble strategy for the two asRNA inserts using the BioBrick assembly system. The digested fused AS insert#1 and #2 were inserted into the pMKN101. Enzymatic activities of β-galactosidase and alkaline phosphatase were measured in 1 mM IPTG and 0.2% L-arabinose-induced MG1655 P_{CP18}-*araE phoA* mt1 cell extracts, expressing asRNAs targeting *lacZ* (B) and *phoA* (C), respectively. All values represent mean ± s.d. (n = 3). Each graph represents at least three independent experiments.

(TATAATG) to generate pMKN104 plasmid (Fig. 4A). We confirmed that transcriptional products from the P_{BAD} promoter of pMKN104, induced with 0.2% arabinose, were approximately five-fold higher than that of pMKN101 (Fig. 4B). The −35 box sequences were also changed to the consensus sequence (TTGACA) in the pMKN104; however, the plasmid presented a leaky expression in the absence of arabinose induction (data not shown). Then, we constructed a pMKN104-based synthetic asRNA expression system to target *lacZ* (ASlacZU) and *phoA* (ASphoAU), and their effects on gene expression were examined. We found that ASlacZU asRNA caused greater repression of β-galactosidase expression by 98.6% in cells harboring pMKN104-ASlacZU compared to that by pMKN101-ASlacZU (Fig. 4C). We also found that ASphoAU asRNA greatly repressed alkaline phosphatase expression by 92.7% in cells harboring pMKN104-ASphoAU compared to that by pMKN101-ASphoAU (Fig. 4D). These results suggest that the effect of gene silencing can be further enhanced by higher dosage of asRNAs transcribed from the strong P_{BAD} promoter.

3.7. Concluding remarks

In this study, we investigated the design and construct of an asRNA expression platform that can repress the expression of target genes using *E. coli lacZ* and *phoA* genes as a model. We defined regions to be targeted, Hfq protein requirement, effects of upstream uORF sequence and Hfq-binding motif sequence, and strength of the promoter that transcribes the asRNA, allowing a sophisticated design for an efficient gene silencing system. It was proven that the RNA chaperon Hfq was not required for this system. These considerations suggest that it would be useful to perform gene silencing

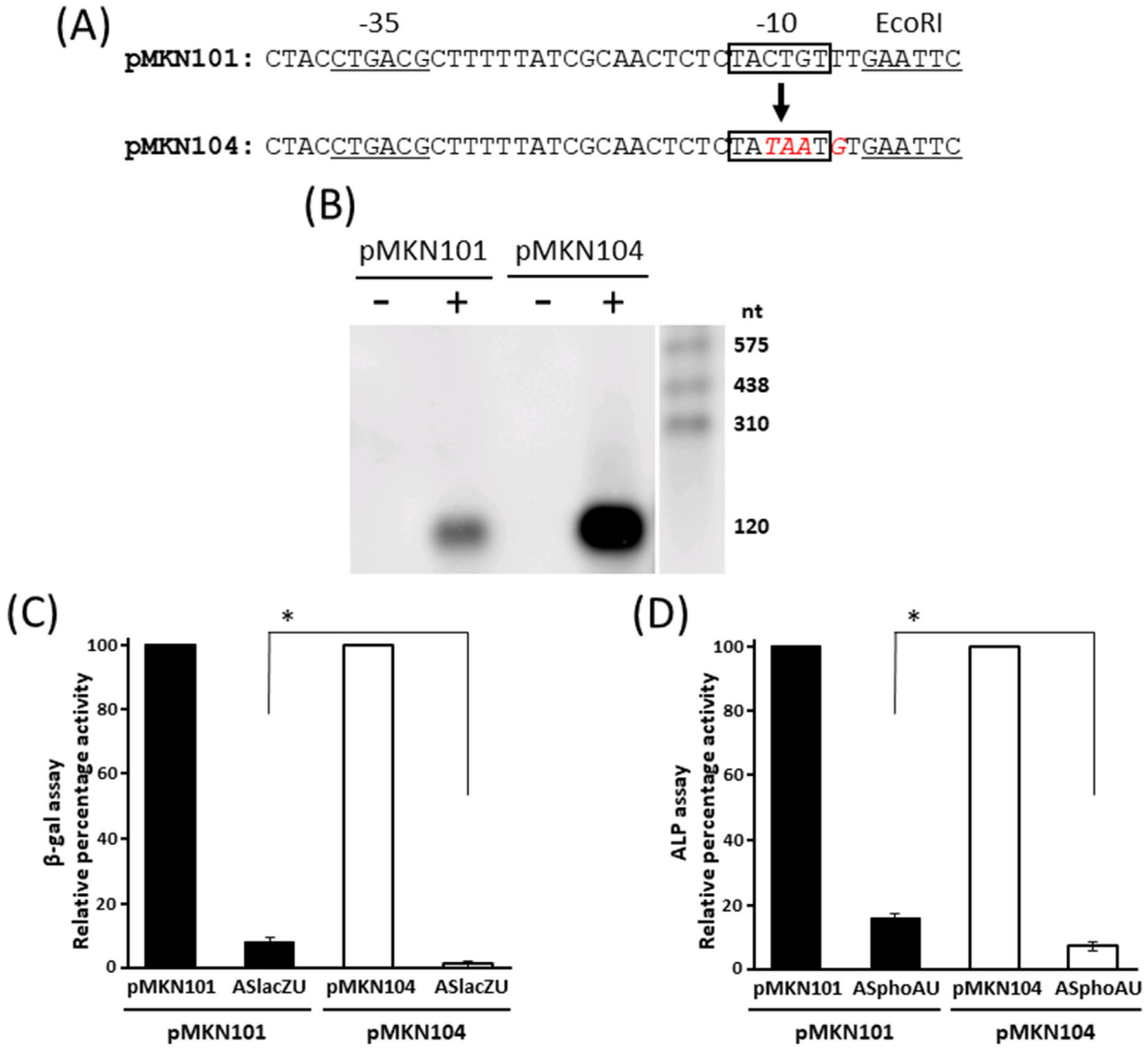


Fig. 4. Gene silencing activities of the synthetic asRNA with mutations in the P_{BAD} -10 promoter region of pMKN101. (A) The mutated sequences in the −10 promoter region are shown in italic and colored in pMKN104. The −35 promoter sequences and *Eco*RI recognition sequences are underlined and the −10 promoter sequences are boxed. (B) Northern blot analysis of arabinose-induced transcript from pMKN101 and pMKN104. Total RNA of DH5α strain harboring pMKN101 or pMKN104 were collected 1 h after adding distilled water (−) or 0.2% L-arabinose (+) at OD_{600} ~0.3. Enzymatic activities of β-galactosidase and alkaline phosphatase were measured in MG1655 P_{CP18}-*araE* with 1 mM IPTG and MG1655 P_{CP18}-*araE phoA* mt1 cell extracts, respectively, harboring the pMKN101 (black bar) or the pMKN104 (white bar), expressing asRNAs (ASlacZU and ASphoAU) targeting *lacZ* (C) and *phoA* (D), respectively. All values represent mean ± s.d. (n = 3). Each graph represents at least three independent experiments. (*$p < 0.05$).

experiments using the P_{BAD} promotor engineered pMKN104 plasmid, which contains a simple 24-nt asRNA sequence binding upstream from the start codon of the target gene. We suggest that the inhibition level can be manipulated for target genes by altering the asRNA abundance. Furthermore, we created a multiplex asRNA expression tool that can knock down two or more target genes using a single plasmid, one promoter, one inducer, and one independent transcript. Thus, this multiplex system enables rapid construction of an asRNA gene cassette and avoids the incompatibility of plasmids and the use of multiple antimicrobial markers and antibiotics, which was less costly and time-consuming than multiple gene silencing using previously reported multiple plasmid asRNA systems. We believe our asRNA-mediated gene silencing system will contribute to broad applications, such as metabolic engineering, synthetic biology, and asRNA-based therapies [21], and can also be applied to dynamic studies on the function of essential and nonessential bacterial genes that are not possible with knockout experiments in a variety of microorganisms.

Declaration of competing interest

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2021.03.152.

Author contribution statement

MK conceived and designed the experiments. MK, SF, YT, and YM performed the experiments. MK wrote the paper. MS supported the study.

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