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枯草芽孢杆菌 168 新型转录终止子的构建与表征

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摘 要：转录终止子作为一种位于终止密码子后的调控信号，负责终止 DNA 的转录和 RNA 的释放。文中首次改造并分析了来源于噬菌体的 λt_0 终止子的发卡结构与富含尿嘧啶的序列对枯草芽孢杆菌 168 中基因转录终止效率以及 mRNA 稳定性的影响。结果表明，相对于野生型的 λt_0 终止子，突变体 M3、M11 和 M12 表现出了更高的转录终止效率，突变体 M3、M4 和 M11 更有利于上游绿色荧光蛋白 mRNA 的稳定。另外，我们发现插入 RNase 作用位点同样提高了 mRNA 的稳定性。研究结果表明终止子中的发卡环对转录终止不是必需的，同时，结果也证明了转录终止子可以作为一种潜在的工具用于提高枯草芽孢杆菌中 mRNA 的稳定性以及相应酶的表达。

关键词：合成生物学，转录终止子，λt_0 终止子，mRNA 稳定性，枯草芽孢杆菌

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Engineering and characterization of new intrinsic transcriptional terminators in *Bacillus subtilis* 168

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Abstract: Terminators as regulatory signals are typically placed behind the last coding sequence to block the transcription of DNA to RNA and release the transcript. In the present study, the hairpin and the U-rich sequence of the bacteriophage λt_o terminator were first modified to investigate their effects on termination efficiency and mRNA stability in *Bacillus subtilis* 168. Compared with the native λt_o terminator, the terminator variants M3, M11 and M12 showed higher termination efficiency values. Moreover, the variantsM3, M4 and M11 showed significant positive effects on the mRNA stability of the upstream *gfp* gene. Additionally, insertion of RNase site also increased the mRNA stability. The results of this study suggested that the composition of the hairpin loop is not required for effective intrinsic termination in *B. subtilis*. Our results also showed that the terminator could also be used as a potential tool for increasing mRNA stability and the corresponding enzyme production in *B. subtilis*.

Keywords: synthetic biology, transcription terminator, λt_o terminator, mRNA stability, *Bacillus subtilis*

Introduction

Terminators are usually located at the end of a gene or an operon where they terminate the transcription of DNA to RNA and release the transcript[1]. Terminators fall into two categories: 1) rho-independent (or intrinsic) terminators and 2) rho-dependent terminators[2-3]. A majority of previous studies focused on intrinsic terminators, due to their function in the dissociation of transcription complexes without the assistance of auxiliary factors. In bacteria, the intrinsic terminator contains a stable GC-rich hairpin stem loop and a U-stretch that required for disturbing the stable transcription elongation complexes[3-5]. The transcription termination process involves in dissociation of RNA polymerase and separation of the RNA:DNA hybrid [6].

For a long time, terminators have not received enough attention as genetic regulators [2] and most studies have focused on prediction and identification alone[2,7], as well as the mutation and evaluation of the effects of the GC-rich hairpin and the poly(U) sequence[4,8-12]. Recently, terminators have been demonstrated to be highly important not only on downstream gene protection against transcript read-through but also for its positive effect on the stabilization of upstream mRNA transcripts [8,13]. In this regard, Chen *et al*. built a library of 582 natural and synthetic terminators. After calculation and comparison, they found that in addition to receiving the greatest contribution from the U-tract, the base content of the hairpin stem also correlated with the terminator strength. Specially, they found that strong terminators usually have higher GC content at the bottom of the hairpin stem. In contrast, no or weak correlations were observed between the hairpin stability and its length [14]. Similarly, Cambray *et al*. characterized 61 natural and synthetic terminators

and attested their roles in the quantitative modeling of transcription termination for the optimization of synthetic genetic systems[8]. More recently, Li *et al.* quantified the relationship between terminator position and terminator efficiency and provided a simple method for fine tuning termination efficiency without changing terminator sequences[11]. In addition to these fundamental studies, many studies have extended the usability of terminators in the construction of genetic systems (such as Logic gates [15-16] and genetic bandpass filter[17-18]), pathway optimization[19] and enzyme expression[9,13,20], suggesting the potential powerful applications of terminators in synthetic biology.

To date, most studies have concentrated on *E. coli* strains and only a small fraction of literatures are focused on other microorganisms[21], such as *Bacillus subtilis*[1,22-23]. In fact, the generally recognized safe strain *B. subtilis* has been widely used as a powerful cell factory for multiple applications, including enzyme production, pathway engineering and synthetic biology[24-27]. Hence, engineering and characterization of rho-independent terminators in *B. subtilis* is of great significance. In the present study, the bacteriophage λt_o terminator was engineered and investigated with an experimental system using *B. subtilis*. The results would have important applications in synthetic biology.

1 Materials and methods

1.1 Bacterial strains and plasmids

The strains, plasmids and primers used in this study are listed in Table 1 and Table 2, respectively. *E. coli* strain JM109 (e14$^-$ (mcrA), endA1, recA1, hsdR17 (rk$^-$, mk$^+$), (lac-proAB) lacIqZM15, relA1) used for plasmid construction and molecular manipulation of all genetic parts and *E. coli* strain TOP10 (F$^-$ mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ (ara-leu) 7697 galE15 galK16 rpsL (StrR) endA1 λ), were purchased from Invitrogen. The pUC57-simple (Amp^r, cloning vector in *E. coli*, isolated from *E. coli* strain DH5α, MCS) that harbored the *rfp* gene was obtained from TaKaRa. To perform the assay on the genetic system in this study, samples of *B. subtilis* 168 (trpC2) obtained from BGSC and pP43NMK (Amp^r, Kan^r, shuttle expression-secretion vector) were used as expression host and vector, respectively.

1.2 Construction of characterization devices

The development of devices that allow the characterization of our new BioBrick terminator variants was based on construction of the GFP/RFP dual fluorescent system being with the inputs/outputs to the system controlled by the P43 promoter. We started by introducing several additional restriction enzyme recognition sites into the multiple cloning site (MCS) of the pP43NMK expression vector. Firstly, a *gfp* reporter gene fragment was amplified using primers GFP-F/GFP-R (Table 2). The resulting PCR product was digested with *Kpn* Ⅰ and *Pst* Ⅰ and ligated intramolecularly to generate plasmid pP43NMK-*gfp*, which contained successive *Sac* Ⅰ, *Xba* Ⅰ, *Spe* Ⅰ and *Pst* Ⅰ recognition sites downstream *gfp*. Secondly, a fragment of the *rfp* reporter gene was amplified from the pUC57-simple plasmid using primers RFP-F/RFP-R and digested with *Sac* Ⅰ and *Spe* Ⅰ. The resulting PCR product was ligated in pP43NMK-*gfp* plasmid digested with the same enzymes (*Sac* Ⅰ and *Spe* Ⅰ) to generate a control device pP43NMK-*gfp*-*rfp*. The next step was the insertion of a testing terminator genetic sequence which was synthesized and amplified using primers λt_o-F/λt_o-R to generate pP43NMK-*gfp*-λt_o-*rfp*. The construction of an insulated terminator was performed by insertion of new genetic devices (RNase site and strong hairpin) in the testing plasmid by means of amplification of pUC57-simple using primers RNase F/RNase R. The resulting PCR product was immediately mixed with 1 U of *Dpn* Ⅰ to digest the remaining circular plasmid and the product was digested with *Not* Ⅰ then self-ligated to generate the plasmid pUC57-RNase site-λt_o-RNase site-strong

Table 1 Strains and plasmids used in this study

Names	Description	Source
Strains		
E. coli JM109	e14$^-$(mcrA), endA1, recA1, hsdR17 (rk$^-$,mk$^+$), (lac-proAB) lacIqZM15, relA1	Invitrogen
TOP10	F$^-$mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ$^-$	Invitrogen
B. subtilis 168	trpC2	BGSC
B. subtilis gfp-λt$_o$-*rfp*	168 derivate, harboring plasmid pP43NMK-*gfp*-λt$_o$-*rfp*	This work
B. subtilis gfp-rfp	168 derivate, harboring plasmid pP43NMK-*gfp-rfp*	This work
B. subtilis gfp-RNase-λt$_o$-RNase-strong hairpin-*rfp*	168 derivate, harboring plasmid pP43NMK-*gfp*-RNase-λt$_o$-RNase-strong hairpin-*rfp*	This work
B. subtilis-gfp	168 derivate, harboring plasmid pP43NMK-*gfp*	This work
B. subtilis-M1	168 derivate, harboring plasmid pP43NMK-M1	This work
B. subtilis-M2	168 derivate, harboring plasmid pP43NMK-M2	This work
B. subtilis-M3	168 derivate, harboring plasmid pP43NMK-M3	This work
B. subtilis-M4	168 derivate, harboring plasmid pP43NMK-M4	This work
B. subtilis-M5	168 derivate, harboring plasmid pP43NMK-M5	This work
B. subtilis-M6	168 derivate, harboring plasmid pP43NMK-M6	This work
B. subtilis-M7	168 derivate, harboring plasmid pP43NMK-M7	This work
B. subtilis-M8	168 derivate, harboring plasmid pP43NMK-M8	This work
B. subtilis-M9	168 derivate, harboring plasmid pP43NMK-M9	This work
B. subtilis-M10	168 derivate, harboring plasmid pP43NMK-M10	This work
B. subtilis-M11	168 derivate, harboring plasmid pP43NMK-M11	This work
Plasmids		
pP43NMK	Amp^r , Kan^r, Shuttle expression-secretion vector	[28]
PUC57- simple	Amp^r, cloning vector in *E. coli*, isolated from *E. coli* strain DH5α, MCS	TaKaRa
pP43NMK-*gfp*-λt$_o$-*rfp*	pP43NMK derivative with gene *gfp* downstream P43 and followed by λt$_o$ terminator upstream *rfp* gene	This work
pP43NMK-*gfp-rfp*	pP43NMK derivative with *gfp* gene following P43 and followed by *rfp* gene	This work
pP43NMK-*gfp*-RNase-λt$_o$-RNase-strong hairpin-*rfp*	pP43NMK derivative with *gfp* gene following P43 and followed by insulated λt$_o$ terminator by RNase E site, strong hairpin and then *gfp* gene	This work
pP43NMK-*gfp*	pP43NMK derivative with *gfp* gene following P43	This work
pP43NMK-M1	pP43NMK derivative with *gfp* gene downstream P43 and followed by λt$_o$ terminator modified in T-tail by A to T and followed by upstream *rfp* gene	This work
pP43NMK-M2	pP43NMK derivative with *gfp* gene downstream P43 and followed by λt$_o$ terminator modified in the loop by UGCC to TTCG and followed by upstream *rfp* gene	This work
pP43NMK-M3	pP43NMK derivative with *gfp* gene downstream P43 and followed by λt$_o$ terminator modified in the loop by UGCC to TTCG , A to T in T-tail and followed by upstream *rfp* gene	This work
pP43NMK-M4	pP43NMK derivative with *gfp* gene downstream P43 and followed by λt$_o$ terminator modified in the loop by UGCC to GAAA and followed by upstream *rfp* gene	This work
pP43NMK-M5	pP43NMK derivative with *gfp* gene downstream P43 and followed by λt$_o$ terminator modified in the loop by UGCC to GAAA, A to T in T-tail and followed by upstream *rfp* gene	This work
pP43NMK-M6	pP43NMK derivative with *gfp* gene downstream P43 and followed by λt$_o$ terminator modified in stem by T to C and followed by upstream *rfp* gene	This work
pP43NMK-M7	pP43NMK derivative with *gfp* gene downstream P43 and followed by λt$_o$ terminator modified in the stem by T to C and T to C closer the loop+ GAAA and followed by upstream *rfp* gene	This work
pP43NMK-M8	pP43NMK derivative with *gfp* gene downstream P43 and followed by λt$_o$ terminator modified in the stem by T to C and T to C closer the loop+ TTCG and followed by upstream *rfp* gene	This work
pP43NMK-M9	pP43NMK derivative with *gfp* gene downstream P43 and followed by λt$_o$ terminator modified in the stem by T to C and T to C closer the loop+ GAAC and followed by upstream *rfp* gene	This work
pP43NMK-M10	pP43NMK derivative with *gfp* gene downstream P43 and followed by λt$_o$ terminator modified in the stem by T to C and T to C closer the loop+ AAATC and followed by upstream *rfp* gene	This work
pP43NMK-M11	pP43NMK derivative with *gfp* gene downstream P43 and followed by λt$_o$ terminator modified in the stem by T to C and T to C closer the loop+ AAAA and followed by upstream *rfp* gene	This work

Table 2 Primers used in this study

Primer name	Primer sequence (5′–3′)
GFP-F	GGGGTACCCTCGAGAAAGGAGGTGAAATGTACACATG
GFP-R	AACTGCAGACTAGTTCTAGAAGTACTTTATTTGTATAGTTCATCCATGCC
RFP-F	ATAAGAATGCGGCCGCAAGAGAGGAATGTACACATGGCCTCCTCCGAGAAC
RFP-R	ATAAGAATGCGGCCGCTGCATAAAAAACGCCCGGCGGCAACCGAGCGTTCT
λto-F	CGAGCTCTGATTAATTAATTCAGAACGCTCG
λto-R	GGACTAGTTTACAGGAACAGGTGGTGGCGGCC
M1-F	ATAAGAATGCGGCCGCTGCAAAAAAAAACGCCCGGCGGCAACCGAGCGTTCT
M2-F	ATAAGAATGCGGCCGCTGCATAAAAAACGCCCGGCCGAAACCGAGCGTTCT
M3-F	ATAAGAATGCGGCCGCTGCAAAAAAAACGCCCGGCCGAAACCGAGCGTTCT
M4-F	ATAAGAATGCGGCCGCTGCAAAAAAAACGCCCGGCTTTCACCGAGCGTTCT
M5-F	ATAAGAATGCGGCCGCTGCATAAAAAACGCCCGGCTTTCACCGAGCGTTCT
M6-F	ATAAGAATGCGGCCGCTGCATAAAAAACGCCCGGCGGCAACCGGGCGTTCT
M7-F	CGAGCTCTGATTAATTAATTCAGAACGCCCGGCGAAAGCCG
M8-F	CGAGCTCTGATTAATTAATTCAGAACGCCCGGCTTCGGCCG
M9-F	CGAGCTCTGATTAATTAATTCAGAACGCCCGGCUAACGCCGGGCGTTTTTTATGCA
M10-F	CGAGCTCTGATTAATTAATTCAGAACGCCCGGCAAATCGCCGGGCGTTTTTTATGCA
M11-F	CGAGCTCTGATTAATTAATTCAGAACGCCCGGCAAAAGCCGGGCGTTTTTTATGCA
RNase-F	AAGGAAAAAAGCGGCCGCGAATTAATTAATCAAAGGAGATCAATACAAATAAT
RNase-R	AAGGAAAAAAGCGGCCGCATTATTTGTATTGATCTCCTTTACTATCTCTCGA

hairpin. From the resulting plasmid, we amplified the fragment harboring the RNase site, λt_o terminator and strong hairpin with primers λt_o-F/λt_o-R. The resulting PCR fragment was digested with *Sac* Ⅰ and *Spe* Ⅰ, then ligated downstream of the *gfp* reporter in pP43NMK-*gfp* digested with the same enzymes to generate the testing device pP43NMK-*gfp*-RNase site-λt_o-RNase site-strong hairpin-*rfp*. Furthermore, we attained our characterization construct model by constructing terminator variants M1, M2, M3, M4, M5, M6, M7, M8, M9, M10 and M11 using primers M1-F/λt_o-R, M2–F/λt_o-R, M3-F/λt_o-R, M4-F/λt_o-R, M5-F/λt_o-R, M6-F/λt_o-R, M7-F/λt_o-R, M8-F/λt_o-R, M9-F/λt_o-R, M10-F/λt_o-R and M11-F/λt_o-R to generate recombinant plasmids named pP43NMK-*gfp*-M1-*rfp*, pP43NMK-*gfp*-M2-*rfp*, pP43NMK-*gfp*-M3-*rfp*, pP43NMK-*gfp*-M4-*rfp*, pP43NMK-*gfp*-M5-*rfp*, pP43NMK-*gfp*-M6-*rfp*, pP43NMK-*gfp*-M7-*rfp*, pP43NMK-*gfp*-M8-*rfp*, pP43NMK-*gfp*-M9-*rfp*, pP43NMK-*gfp*-M10-*rfp* and pP43NMK-*gfp*-M11-*rfp*, respectively. Recombinant bacteria harboring controls and testing devices were isolated, and the entire resulting devices were confirmed by sequencing. We further continued by transforming all confirmed plasmids into *B. subtilis* 168 competent cells, and finally we obtained a control strain *B. subtilis* 168 GFP-RFP and recombinant test strains *B. subtilis* 168-M1, *B. subtilis* 168-M2, *B. subtilis* 168-M3, *B. subtilis* 168-M4, *B. subtilis* 168-M5, *B. subtilis* 168-M6, *B. subtilis* 168-M7, *B. subtilis* 168-M8, *B. subtilis* 168-M9, *B. subtilis* 168-M10, *B. subtilis* 168-M11 and *B. subtilis* 168 GFP-RNase site-λt_o-RNase site-strong hairpin-*rfp* which were treated and subjected to the characterization experiment.

1.3 Media and culture conditions

E. coli JM109, TOP10 and *B. subtilis* 168 were typically cultured in LB broth (per liter: 10.0 g tryptone, 5.0 g yeast extract, and 10.0 g NaCl) at 37 ℃ with shaking speed of 220 r/min. Solid media were prepared by adding 1.5 g/L agar to the respective media. Unless otherwise stated, the antibiotic concentrations were 100 mg/L ampicillin for *E. coli*, and 100 mg/L kanamycin for *B. subtilis* 168. To evaluate the expression of our genetic devices, all plasmids harboring genetic sequences were cultured in LB medium supplemented with antibiotic and flask cultivations were performed at 37 ℃ with a rotational speed of 220 r/min for 12 hours where samples were collected and treated every 2 hours and subjected to fluorescence measurements.

1.4 Analytical methods

1.4.1 GFP and RFP intensity quantification

For the GFP and RFP determination, the culture broth was centrifuged at 10 000×*g* for 10 min, washed

with 0.1 mol/L phosphate-buffered saline (pH 7.4), and diluted in the same buffer. Therefore, 200 μL of each sample was pipetted, added to the 96-well plates and immediately subjected to fluorescence determination. The GFP and RFP fluorescence intensity produced by the characterization devices were measured by using a Cytation 3 Cell Imaging Multi-Mode Reader (BioTek) at an excitation/emission wavelength of 490/530 nm and 555/584 nm respectively.

1.4.2 Analysis of GFP mRNA transcript level

Samples taken from the culture media between 7 and 8 hours were thawed and centrifuged for 2 min at~11 000×*g* at 4 ℃. The supernatant was discarded, and the pellet was immediately frozen at −80 ℃. Cell pellets were suspended in Tris-EDTA (TE) buffer (10 mmol/L Tris-HCl, pH 8.0, 1 mmol/L EDTA) with lysozyme to a final concentration of 0.4 mg/mL. Total RNA was extracted with RNAprep Pure Kit (Tiangen biotech, CHINA) and RNA concentration was determined with the NanoDrop1000 (Thermo Fisher Scientific, MA, USA). Furthermore, cDNA was synthesized by TaKaRa Prime Script Reverse Transcriptase with gDNA eraser and quantitative-PCR (Q-PCR) was carried out using TaKaRa SYBR Premix Dimer Eraser.

2 Results and discussion

2.1 Structures of the λt_o terminator and its variants

The DNA sequences as well as the free energies of the λt_o terminator [28] and its variants are shown in Table 3. In particular, both the RNA hairpin structures and their corresponding free energies were predicted by *KineFold* [29]. The loop and the adjacent C-G base pair were examined and mutated since each contributed to the terminator strength[9]. Generally, nucleotides mismatches occur very rarely in strong terminators, thus, the nucleotides mismatches in the stem of the hairpin were eliminated. In addition, the effects of the poly (T) tail was also analyzed. Specifically, the TGCC loop in variants M2, M3, M4, M5, M6, M7 and M8 were replaced with TTCG and GAAA which are considered to increase terminator efficiency and mRNA stability in many prokaryotes [7]. In variants M1, M2, M3 and M4, A was substituted with T to increase the number of T in the poly (T) tract but in variants M9, M10 and M11, the TGCC loop was substituted with GAAC, AAATC and AAAA, respectively. Additionally, to eliminate the mismatched nucleotide pair, T at positions 22 and 26 in the G+C rich stem were substituted with C in the variants M6, M7, M8, M9, M10 and M11 (Fig. 1).

2.2 Termination efficiencies of the constructed terminator variants

To assess the effects of the loops, poly (T) tract and mismatched nucleotide pair on transcription termination, as well as the termination efficiencies of the variants were determined by measuring the input and output fluorescence of the reporter genes (*gfp* and *rfp*) in the constructed system (Fig. 2). As

Table 3 Wild type and terminator variant sequences used in this study

Names	Wild-type and terminators variants DNA sequences	Free energy (kcal/mol)
WT λt_o	TGATTAATTAATTCAGAACGCTCGGTTGCCGCCGGGCGTTTTTTATGCA	−17.5
M1	TGATTAATTAATTCAGAACGCTCGGTTGCCGCCGGGCGTTTTTTTTGCA	−17.5
M2	TGATTAATTAATTCAGAACGCTCGGTTTCGGCCGGGCGTTTTTTTTGCA	−19.3
M3	TGATTAATTAATTCAGAACGCTCGGTTTCGGCCGGGCGTTTTTTTTGCA	−19.3
M4	TGATTAATTAATTCAGAACGCTCGGTGAAAGCCGGGCGTTTTTTTTGCA	−20.4
M5	TGATTAATTAATTCAGAACGCTCGGTGAAAGCCGGGCGTTTTTTATGCA	−20.4
M6	TGATTAATTAATTCAAAACGCCCGGCGAAAGCCGGGCGTTTTTTATGCA	−21.7
M7	TGATTAATTAATTCAGAACGCCCGGCGAAAGCCGGGCGTTTTTTATGCA	−24.3
M8	TGATTAATTAATTCAGAACGCCCGGCTTCGGCCGGGCGTTTTTTATGCA	−23.5
M9	TGATTAATTAATTCAGAACGCCCGGCGAACGCCGGGCGTTTTTTATGCA	−22.0
M10	TGATTAATTAATTCAGAACGCCCGGCAAATCGCCGGGCGTTTTTTATGCA	−22.0
M11	TGATTAATTAATTCAGAACGCCCGGCAAAAGCCGGGCGTTTTTTATGCA	−21.5

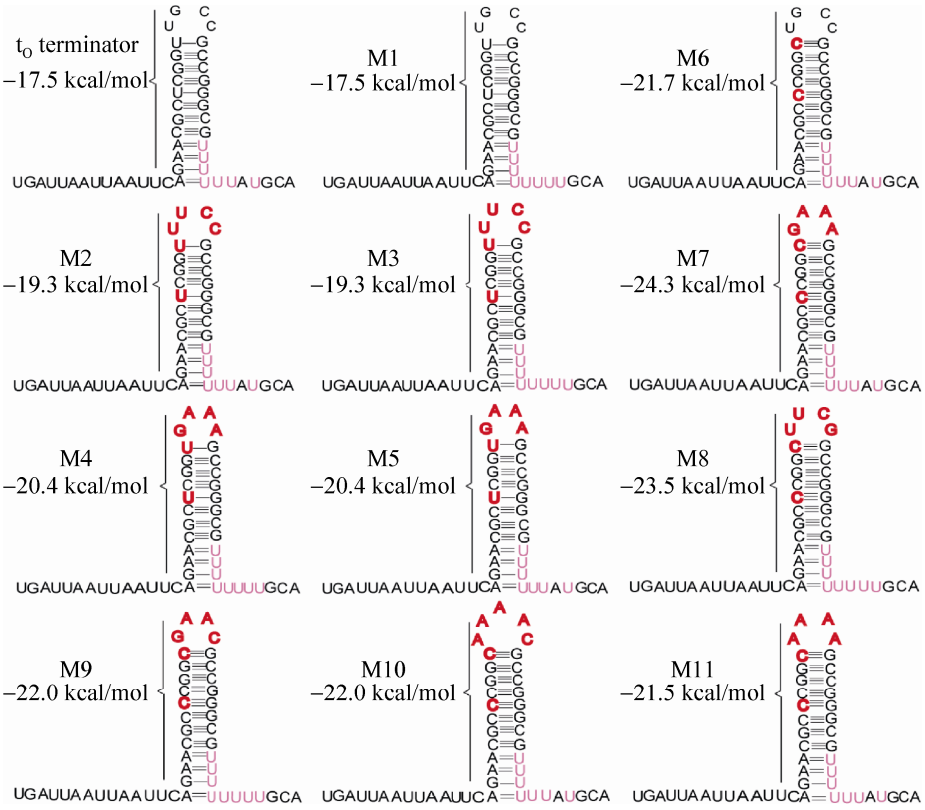

Fig. 1 Secondary structures of the λt_O terminator and its variants. The predicted stabilities of the RNA hairpin structures were determined by the *Kinefold* web server. The termination positions for all engineered functional λt_O variants were highlighted in red.

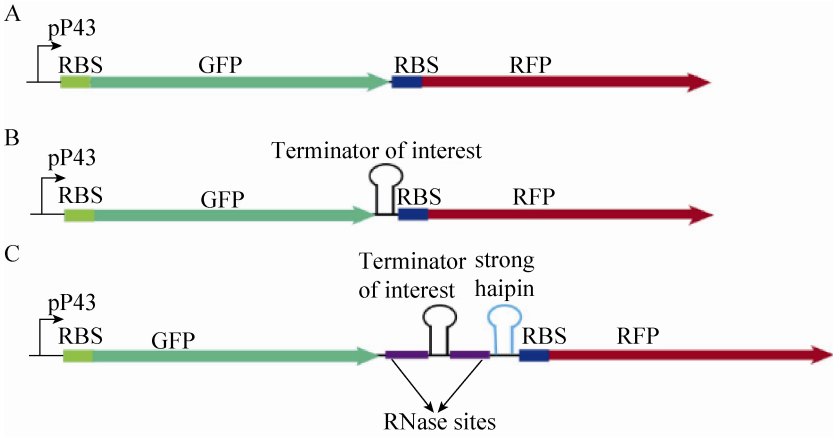

Fig. 2 Structures of the expression cassettes with two reporter genes *gfp* and *rfp*. (A) The control without terminator. (B) Terminator was inserted between two fluorescent protein encoding genes. (C) RNase sites and strong hairpin were inserted followed by *rfp* gene. RBS: ribosome-binding site.

shown in Fig. 3B, the insertion of the wild-type strong λt_o terminator and its variants resulted in low expression of *rfp*, suggesting the strong termination efficiency of all the terminators. After further quantification of the GFP fluorescence (Fig. 3A) and calculation [18], terminator variants M3, M4 and M11

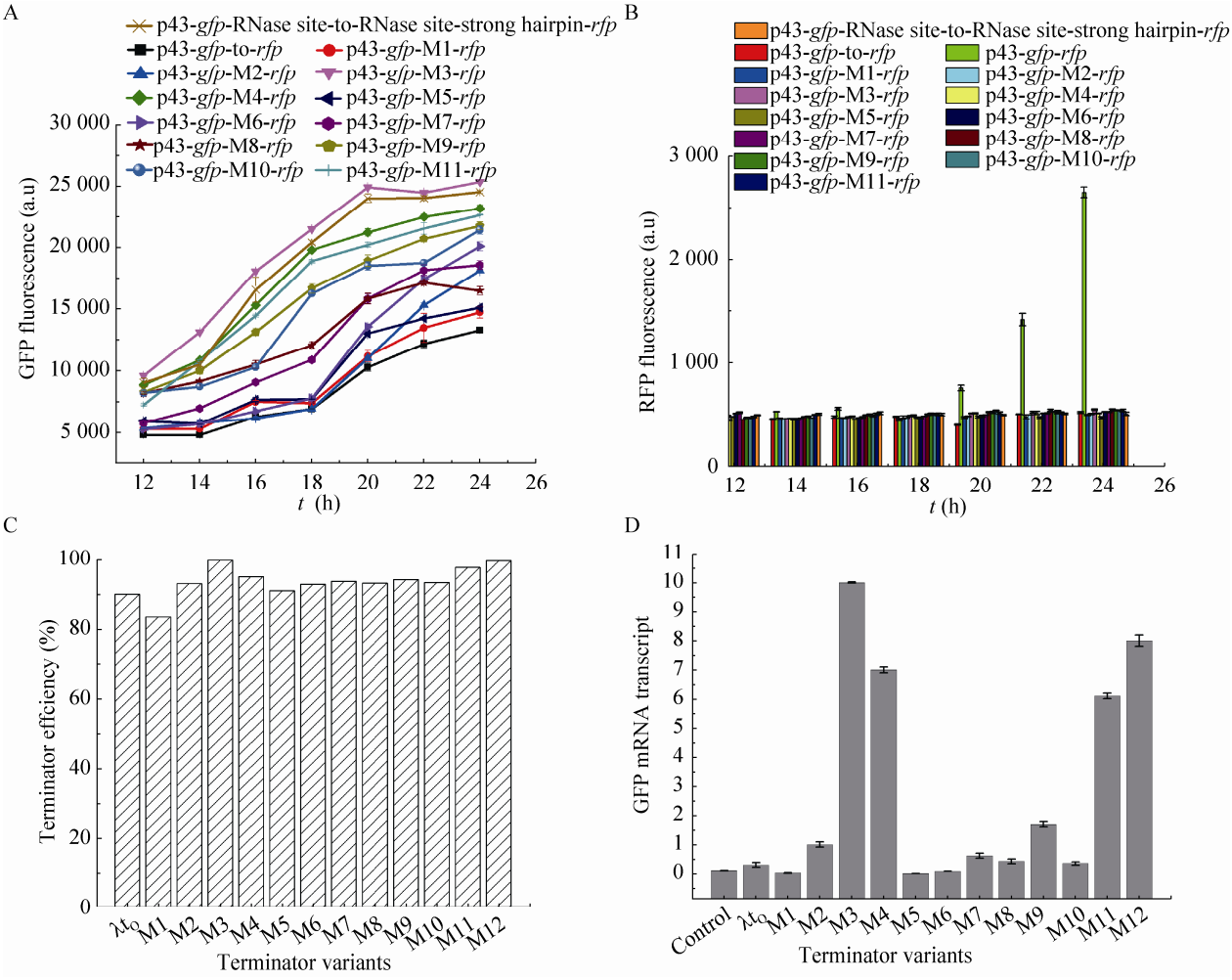

Fig. 3 Comparison of termination efficiency and mRNA stability of the λt_o terminator variant. (A) The GFP fluorescence of the native terminator (control) and the variants. (B) The RFP fluorescence of the native terminator and its variants. (C) Termination efficiencies of native (λt_O) and its variants. (D) The mRNA levels of the *gfp* gene with the λt_O terminator and its variants. Error bars are standard deviations calculated from three independent experiments.

showed higher termination efficiency values compared with the wild type λt_o and the remaining variants (Fig. 3C). Interestingly, the inclusion of RNase sites in the variant M12 (Fig. 3C), showed a considerable termination efficiency of about 98% which prove its positive effect on the termination efficiency. The results clearly showed that the loop causes no significant effect on transcription termination although both tetra loops GAAA and TTCG are frequently found in many prokaryotic and eukaryotic RNAs [2-7] . Meanwhile, the results also suggested that the introduction of mutations to enhance the RNA stem stability did not contribute to the termination efficiency, which are different from that in *E. coli* [22].

2.3 Insertion of terminator increased upstream gene expression

In addition to evaluating the termination efficiency, the effect of the terminators on the

upstream gene was also investigated and compared. Furthermore, the low *gfp* expression revel obtained by measuring the negative control compared with other studied terminator variants (Fig. 3A), explains the role of terminators on the upstream gene. Interestingly, the GFP fluorescence was significantly increased when inserting all the terminator variants (Fig. 3A), indicating its positive effect on the RNA stability of the upstream *gfp*, which is similar to previous studies in *E. coli* [10,30] and *S. cerevisiae*[9,13]. To further confirm this phenomenon, the mRNA of *gfp* in all the constructs were analyzed. Compared with the control, most of the constructs with terminator variants (especially M2, M3, M4, M9, M11 and M12) yielded a higher level of *gfp* mRNA (Fig. 3D). Recently, it has been reported that the addition of RNase sites surrounding the target terminator can impact the expression of reporter genes in *E. coli* [8,31]. Thus, two RNase sites and one strong hairpin were inserted between the reporter genes *gfp* and *rfp* (Fig. 2C). As expected, the expression of *gfp* was significantly increased (Fig. 3A).

3 Conclusions

In this study, the hairpin and the U-rich sequence of the strong bacteriophage λt_o terminator were first engineered and investigated in *Bacillus subtilis* 168. Compared with the wild-type strong λt_o terminator, all the variants showed a higher termination efficiencies. The results suggested that the hairpin loop is not a critical factor for strong terminator. More importantly, the results first demonstrated that terminator insertion can significantly increase the expression of upstream gene in *B. subtilis*. As a result, these rho-independent terminators should be used as a potential tool for synthetic biology research in *B. subtilis*. In addition, the constructed terminators should also be used for increasing mRNA stability and the corresponding enzyme production in *B. subtilis*.

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