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Riboswitches for Enhancing Target Gene Expression in Eukaryotes

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Various RNA elements have been found to act as regulators of gene expression, for example in ribozymes,[1] RNA interference,[2] riboregulators,[3] and riboswitches.[4,5] Inspired by these natural RNAs, new RNA molecules have been engineered as gene regulators with novel biological functions.[6,7] Riboswitches are conserved RNA elements that precisely and specifically sense small molecules to control gene expression, and they have fundamental advantages over other methods for the control of gene expression by external stimuli. The thiamine pyrophosphate (TPP)-dependent riboswitches have been discovered not only in prokayotes but also eukaryotes,[8–11] and the structures and mechanisms of gene regulation for TPP-dependent riboswitches have been determined.[12–17]

In a previous study, we showed that the TPP-dependent riboswitch in the intron of the 5′-untranslated region (UTR) of *thiA* in a eukaryote (*Aspergillus oryzae*) can regulate mRNA splicing.[18] We further reported that physiological concentrations of Mg^{2+} play a critical role in the regulation of TPP binding by the *thiA* riboswitch.[19] Breaker and co-workers also recently discovered a TPP-dependent riboswitch in the filamentous fungus *Neurospora crassa* and demonstrated that it controls alternative splicing.[20] On the basis of these previous reports, we are currently investigating the use of riboswitches in new biotechnologies and attempting to engineer them for novel functions, namely, to upregulate rather than downregulate gene transcription.

Although we previously found that TPP binding to the *thiA* riboswitch causes improper splicing,[18] we did not determine the splicing site. In the current study, to elucidate the mechanism of action and to engineer the riboswitch, we first identified the splicing site of the intron by reverse transcriptase-polymerase chain reaction (RT-PCR) and DNA sequencing. We constructed a promoter-β-glucuronidase (GUS) reporter gene plasmid (pNG*thiA*) containing the 5′-UTR of *thiA*. We used this plasmid to transform *A. oryzae* with pNG*thiA* and then treated the transformed cells with or without 10 μM thiamine. These cells take up thiamine and spontaneously convert it into TPP,[18] allowing activation of the TPP-dependent riboswitch by application of thiamine as an external stimulus.

Total RNA was extracted from the cells and amplified by RT-PCR as described in Figure 1A. Figure 1B shows native polyacrylamide gel electrophoresis (PAGE) of the RT-PCR products. DNA sequencing revealed that mature mRNA was produced only in the absence of thiamine, whereas the level of immature mRNA increased in the presence of thiamine. Moreover, sequencing of the immature mRNA showed clearly that the 5′-splicing site in the absence of thiamine was at position −338 relative to the translation initiation site (+1) (Figure 1C). In contrast, in the presence of thiamine, the 5′-splicing site was at position −253, demonstrating that the immature mRNA contains a 85-base remnant of the intron. The remnant sequence of the intron has three potential translation initiation codons at positions −262, −290, and −299 (Figure 1C), which can induce the frame shifts in the codons, leading to inactivation of proteins. These results are consistent with findings that the alternative translation initiation codons observed in the TPP-dependent riboswitch system in *N. crassa* can reduce protein expression.[20] These results confirm that the *thiA* riboswitch alters the 5′-splicing site upon TPP binding.

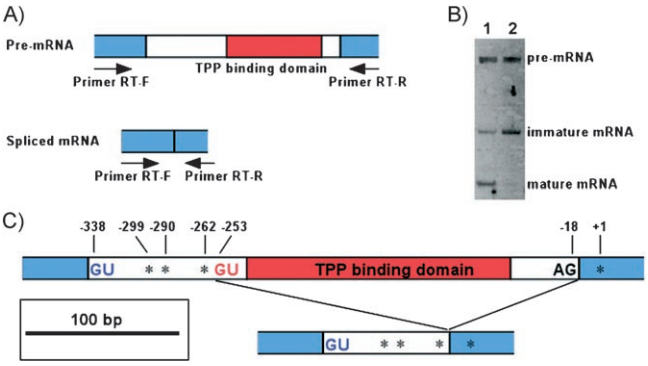

Figure 1. A) Pre-mRNA and spliced mRNA were amplified by RT-PCR with primers RT-F and RT-R. Blue and white rectangles indicate exons and introns, respectively. B) RT-PCR products were separated by native PAGE on a 5% acrylamide gel. Lanes 1 and 2 indicate the PCR products in the absence and presence of thiamine, respectively. C) The 5′-UTR of pre-mRNA and immature mRNA produced from the *thiA* gene. Asterisks indicate putative start codons, and the numbers indicate the sequence position relative to the translation initiation site (+1)

Based on the splicing site and mechanism of the *thiA* riboswitch, we attempted to engineer an "on riboswitch" that can upregulate gene expression in response to thiamine. We specifically focused on the 85-base remnant of the intron that is produced upon splicing in the presence of thiamine. We suspected that removing the remnant sequence would prevent

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improper splicing from proceeding in the presence of thiamine (Figure 2). We therefore first engineered the *thiA* riboswitch E1 in which the 85 bases were removed from the 5′-splicing site of the intron. We then constructed a promoter-GUS reporter

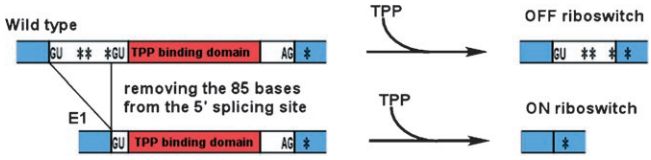

Figure 2. Strategy for controlling gene expression through the engineering of the *thiA* riboswitch. Blue, red, and white rectangles indicate exons, the TPP binding domain, and introns, respectively. Asterisks indicate putative start codons.

gene plasmid containing the E1 riboswitch and analyzed it in the absence and presence of thiamine. The GUS activities of the original *thiA* riboswitch were 3708 ± 15 and 106 ± 1.3 unit mg^{-1} protein, respectively, demonstrating the downregulation of the natural riboswitch. On the other hand, the GUS activities of engineered E1 riboswitch were 357 ± 6.2 and 450 ± 12 unit mg^{-1} protein, respectively. Although the response to exogenous thiamine was altered, the difference in GUS activities of the E1 riboswitch was insufficient for use in gene regulation.

We next attempted to improve the extent of upregulation mediated by the riboswitch. In fungi, there is a very strong correlation between the total length of an intron and the length from the 5′-splicing site to the branch point.[21] As the TPP-binding domain of the *thiA* riboswitch is located between the 5′-splicing site and the branch point, the length between the 5′-splicing site and the TPP-binding domain could be important for the splicing reaction. We therefore truncated the sequence between the 5′-splicing site and the TPP-binding domain of the E1 riboswitch generating the E2, E3, and E4 riboswitches (Figure 3 A), which have different lengths between the 5′-splicing sites and the TPP binding domain. We then inserted these new riboswitches into promoter-GUS reporter gene plasmids. Figure 3 B shows their GUS activities. Surprisingly, the GUS activities of E2 and E3 riboswitches in the presence of thiamine were 4.7 and 4.3 times higher, respectively, than the activities in the absence of thiamine, whereas the E4 riboswitch construct had almost no GUS activity. Although the length should be further optimized, these alternations in gene expressions are comparable to the difference in gene expression mediated by the naturally occurring TPP-dependent riboswitch in *N. crassa*, wherein gene expression in the presence of thiamine is about four times higher than that in its absence.[20]

We next performed RT-PCR analysis to confirm the sequences of the spliced products produced from the engineered riboswitches. Figure 3 C shows native PAGE results of the amplified DNAs derived from the transcripts. The bands were extracted from the gel for sequencing. We found that the 5′-splicing site of E1 riboswitch was nine bases downstream of the expected splicing site, leading to a nine base remnant of the intron so that the lower bands produced by the E1 riboswitch migrated slower than that produced by the *thiA* riboswitch. Although the reason for the remaining nine bases is unclear, the band intensity of the spliced mRNA from the E1 riboswitch was the same in the presence and absence of thiamine, indicating the lack of an effect of thiamine. On the other hand, the signals derived from the spliced mRNAs of riboswitches E2 and E3 in the presence of thiamine were more intense than those in its absence, indicating that the mRNA was spliced more efficiently in the presence of thiamine. In addition, the spliced mRNA produced by the E4 riboswitch was not detected by RT-PCR analysis. These RT-PCR and DNA sequencing results are consistent with the GUS activities, demonstrat-

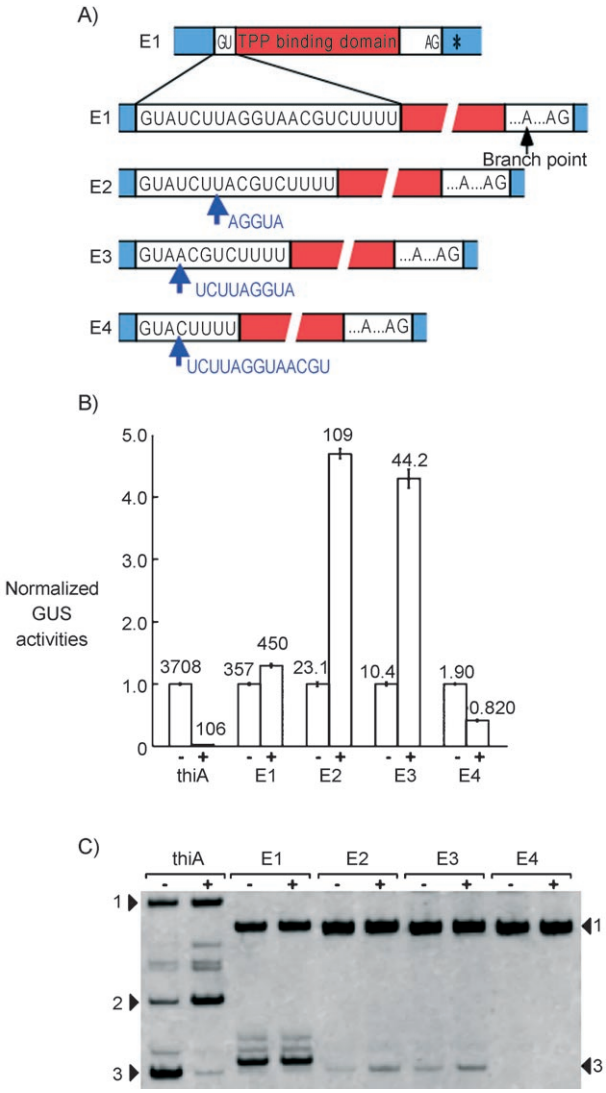

Figure 3. A) E2–E4 riboswitches were designed by truncating the E1 riboswitch. The blue sequences and arrows indicate the truncated sequence and position, respectively. B) GUS activity of the engineered riboswitches in the absence (−) and presence (+) of thiamine. The GUS activity of the riboswitches in the absence of thiamine was normalized to 1.0. The actual GUS activities (unit mg^{-1} protein) are indicated at the top of each bar. C) Splicing of engineered *thiA* pre-mRNA without (−) or with (+) thiamine. Spliced mRNA and substrate pre-mRNA were detected by RT-PCR using primers RT-F and RT-R (Figure 1 A), and PCR products were separated by native PAGE on a 5% acrylamide gel. Bands 1, 2, and 3 indicate pre-mRNA, immature mRNA, and mature mRNA, respectively.

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ing that the E2 and E3 riboswitches act as "on riboswitches" that are activated by thiamine.

To confirm the gene regulation mediated by the engineered riboswithches as revealed by the GUS reporter assay and RT-PCR analysis, it is important to compare the binding affinities of the riboswitches for TPP, which is spontaneously generated from thiamine in vivo. Figure 4 shows the normalized GUS activities of the *thiA* and E2 riboswitches in the presence of various concentrations of thiamine. The apparent association constant for thiamine binding to these riboswitches at 30 °C were $(3.2 \pm 1.1) \times 10^6$ and $(3.5 \pm 0.6) \times 10^6\ \text{M}^{-1}$, respectively. The affinities of these two riboswitches for thiamine were identical, suggesting that the engineered riboswitch retained normal TPP binding.

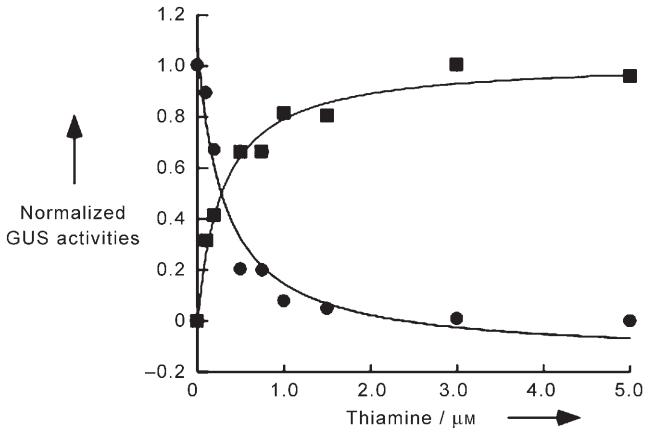

Figure 4. GUS activity of the *thiA* (circles) and E2 (squares) riboswitches at different thiamine concentrations. Because one TPP molecule, which is generated spontaneously from thiamine in vivo, binds to each riboswitch,[19] the data from the *thiA* and E2 riboswitches were fitted to the equation below to estimate the equilibrium parameters based on a model that assumes a single binding site:[22]
$\theta = aK_a[\text{thiamine}]/(1+K_a[\text{thiamine}]) + b$
where θ is the normalized GUS activity, K_a is the apparent association constant of thiamine binding, [thiamine] is the molar concentration of thiamine, a is a scale factor, and b is the initial θ value.

In conclusion, we successfully engineered the *thiA* riboswitch from its natural state as an "off riboswitch" into an artificial "on riboswitch". There are many natural and engineered gene expression systems developed to the applications. The most frequently used systems for controlling gene expression by exogenous molecules rely on specific sensor proteins and specialized promoter elements. The TPP-dependent riboswitches, however, do not require such a specialized promoter for the gene regulation because they directly regulate splicing. Therefore, the riboswitches can be embedded into any endogenous gene or engineered vector transcription unit. The inducible gene expression systems can be designed rationally by the engineered riboswitch, like the conventional gene expression systems. Importantly, the riboswitches developed here can upregulate the target gene in eukaryotes; therefore, application of these riboswitches can be adapted to gene regulation in mammals. Because thiamine has very low toxicity in vivo, these "on riboswitches" could be useful in therapeutic applications.

Experimental Section

Construction of promoter-GUS reporter gene plasmids and transformation experiments: The 5′-UTR ranging from −729 to −1 of the *thiA* was amplified from genomic DNA of *A. oryzae* RIB40 by PCR using primers THIA-F (5′-GGCCCGGGGACAGACGGGCAATT-GATTACG-3′) and THIA-R (5′-CCGTCGACGTTTCAAGTTGCAATGAC-3′). PCR using KOD-Plus (Toyobo) was carried out for 2 min at 94 °C, and products were amplified by PCR for 30 cycles of 15 s at 94 °C, 30 s at 55 °C, and 60 s at 68 °C. The amplified DNA products were phosphorylated by TaKaRa BKL Kit (Takara) and inserted into the SmaI site of GUS reporter plasmid pNG1[18] to obtain pNG*thiA*. The E1 riboswitch was constructed from pNG*thiA*. The 5′-site of E1 riboswitch was amplified by PCR using primers THIA-F and E1-R (5′-GACGTTACCTAAGATACATTGTCGGTTGGTTTGG-3′), and the 3′-site of the product was amplified by PCR using primers E1-F (5′-CCAAAC-CAACCGACAATGTATCTTAGGTAACGTC-3′) and THIA-R. Next, the amplified 5′- and 3′-sites were mixed and amplified by PCR using primers THIA-F and THIA-R. The PCR was carried out using KOD-Plus (Toyobo) and incubated for 2 min at 94 °C, followed by 20 cycles for 15 s at 94 °C, 30 s at 55 °C, and 60 s at 68 °C. Riboswitches E2–E4 were constructed using the same method as for the E1 riboswitch. PCR primers were as follows: 5′-site of E2 riboswitch, THIA-F and E2-R (5′-CCAAAGACGTAAGATACATTGTCGGTTGGTTTGG-3′); 3′-site of E2 riboswitch, E2-F (5′-CCAAACCAACCGACAATGTATCT-TACGTCTTTGG-3′) and THIA-R; 5′-site of E3 riboswitch, THIA-F and E3-R (5′-CACGCCAAAGACGTTACATTGTCGGTTGGTTTGG-3′); 3′-site of E3 riboswitch, E3-F (5′-CCAAACCAACCGACAATGTAACGTCTTT-GGCGTG-3′) and THIA-R; 5′-site of E4 riboswitch, THIA-F and E4-R (5′-GGCCCACGCCAAAGTACATTGTCGGTTGGTTTGG-3′); 3′-site of E4 riboswitch, E4-F (5′-CCAAACCAACCGACAATGTACTTTGGCGTGGGCC-3′) and THIA-R. All PCR products were confirmed by DNA sequencing using an ABI PRISM 310 genetic analyzer. The amplified DNA products were phosphorylated using a TaKaRa BKL Kit (Takara) and inserted into the SmaI site of pNG1 to obtain pNGE1, pNGE2, pNGE3, and pNGE4, respectively. *Escherichia coli* DH5α was used as a host during the manipulation of plasmids. Transformation of *A. oryzae* was performed according to Gomi et al.[23]

RT-PCR analysis: Approximately 5×10^7 conidiospores of *A. oryzae* transformants were inoculated into 100 mL of minimal medium (Czapeck Dox medium[24]) and incubated at 30 °C with shaking at 180 rpm for 48 h with or without 10 μM thiamine. Total RNA was prepared using ISOGEN (Nippon gene). One μg of total RNA was reverse-transcribed and amplified using a Takara One Step RNA PCR kit (AMV) (Takara) according to the manufacturer's instructions. PCR primers were as follows: RT-F, 5′-TTCCCAAACCAACCGACAAT-3′; RT-R, 5′-TGATCAATTCCACAGTTTTC-3′. Amplified products were resolved by PAGE on a 5% acrylamide gel in 1 × TBE buffer and stained with GelStar® Nucleic Acid Stain (Takara).

GUS reporter assay: Cell-free extracts were prepared by the method of Tada et al.[25] from mycelia grown under the same conditions as those used for RNA preparation. GUS activity was determined spectroscopically using *p*-nitrophenyl glucuronide as a substrate as described by Jefferson et al.[26]

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