Thiamine-regulated gene expression of *Aspergillus oryzae thiA* requires splicing of the intron containing a riboswitch-like domain in the 5′-UTR

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Abstract Exogenous thiamine regulates *Aspergillus oryzae thiA*, which is involved in thiamine synthesis. One of the two introns in its 5′-untranslated region (5′-UTR) contains motifs (regions A and B) highly conserved among fungal thiamine biosynthesis genes. Deletion of either region relieved the repression by thiamine and thiamine inhibited intron splicing, suggesting that regions A and B are required for efficient splicing. Furthermore, transcript splicing was essential for *thiA* gene expression. These observations suggest a novel gene expression regulatory mechanism in filamentous fungi, in which exogenous thiamine controls intron splicing to regulate gene expression. Interestingly, regions A and B constitute a part of a thiamine pyrophosphate-binding riboswitch-like domain that has been quite recently found in the 5′-UTR of *thiA*.

Key words: Thiamine; Gene regulation; Pyrithiamine; Intron splicing; Riboswitch; *Aspergillus oryzae*

1. Introduction

Pyrithiamine (PT) is a potent antagonist of thiamine and very effectively inhibits the growth of *Aspergillus oryzae*. *A. oryzae* is the most important filamentous fungus for traditional fermentation industries in Japan and has recently attracted attention as a promising host for heterologous protein production by recombinant DNA techniques. However, because *A. oryzae* is highly resistant to a variety of antifungal reagents, few selectable markers have been available for transformation of industrial strains, which have no mutations for any nutrient requirements. We cloned the gene responsible for PT resistance (*ptrA*) from a mutant of *A. oryzae* that is resistant to PT and have developed a transformation system for *A. oryzae* using *ptrA* as a dominant selectable marker [1]. This system can be applicable to a variety of filamentous fungi, including *A. nidulans*, *A. kawachii*, *A. terreus*, *A. fumigatus*, and *Trichoderma reesei* [2,3].

The nucleotide sequence of *ptrA* differs from that of *thiA* (the wild-type allele of *ptrA*) by only a single base substitution in the 5′-untranslated region (5′-UTR): the A at position −68 (relative to the translation initiation site as +1) of *thiA* is replaced by G in *ptrA*. This point mutation is responsible not only for the acquisition of PT resistance but also for the loss of thiamine feedback regulation of *thiA* [1]. Therefore, thiamine-regulated gene expression of *thiA* appears to be controlled through a putative *cis*-element that includes the mutation point of *ptrA* in the 5′-UTR.

We found two motifs (designated regions A and B) in the intron in the 5′-UTR of *thiA* whose nucleotide sequences are highly conserved among microbial genes involved in thiamine biosynthesis. Interestingly, the mutation point of *ptrA* is located in region A. Regions A and B are necessary for efficient intron splicing in the 5′-UTR and thus are essential for thiamine-regulated gene expression. Recently, a riboswitch triggered by thiamine pyrophosphate (TPP) was demonstrated to control thiamine biosynthetic operons in *Escherichia coli* [5,6]. In addition, Sudarsan et al. have quite recently reported putative mRNA elements in filamentous fungi and plants that have the consensus sequence and structure of the TPP-binding riboswitch [7]. Intriguingly, regions A and B are a part of the riboswitch-like domain found in *thiA*.

2. Materials and methods

2.1. Strains, plasmids, and media

A. oryzae niaD300 (*niaD*) [8] was used as a host strain for transformation. The plasmid pGAPG1 [9], which carries a fusion gene consisting of the *E. coli uidA*-encoded β-glucuronidase (GUS) and the *A. oryzae amyB* terminator (*TamyB*), and pUN1 [10], which contains an *niaD* gene, were used to construct promoter–GUS reporter gene plasmids. *E. coli* DH5α was used as a host during the manipulation of plasmids. Czapek-Dox (CD) medium and DPY medium [1] were used as minimal and complete media, respectively.

2.2. Construction of promoter–GUS reporter gene plasmids

The *uidA-TamyB* fusion gene was amplified by polymerase chain reaction (PCR) using pGAPG1 as a template and primers 5′-GGTCGACATGTTACGTCCTGTAGAAACCCC-3′ (inserted *Sal*I site underlined) and 5′-CCGCATGCCTTTCCTATAATAGACTAGCG-3′ (inserted *Sph*I site underlined), corresponding to the 5′-terminus of the *uidA* coding region and the 3′-terminus of *TamyB*, respectively. The PCR product was digested with *Sal*I and *Sph*I and inserted into the *Sal*I-*Sph*I gap of pUN1 to obtain pNG1.

PthiA, consisting of the 5′-UTR ranging from −729 to −1 of *thiA*, was amplified from genomic DNA of *A. oryzae* RIB40 by PCR using the forward primer 5′-GGCCCGGGGACAGACGGGCAATTGATTACG-3′ (inserted *Sma*I underlined) and the reverse 5′-CCGTCGACGTTTCAAGTTGCAATGAC-3′ (inserted *Sal*I site under-

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Abbreviations: 5′-UTR, 5′-untranslated region; GUS, β-glucuronidase; PT, pyrithiamine; TPP, thiamine pyrophosphate

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lined). The amplified DNA fragment was digested with *Sma*I and *Sal*I and inserted into the *Sma*I-*Sal*I gap of pNG1 to obtain pNGTH.

Various deletions and substitutions of nucleotides were introduced into *PthiA* by PCR site-directed mutagenesis [11].

2.3. Transformation experiments

Transformation of *E. coli* was performed according to Hanahan et al. [12] and of *A. oryzae* by the method of Gomi et al. [13].

2.4. Northern blot analysis

Approximately 5×10^7 conidiospores of *A. oryzae* transformants were inoculated into 100 ml of CD and incubated at 30°C with shaking at 180 rpm for 24 h. Total RNA was prepared by the method of Cathala et al. [14]. Ten μg of total RNA was loaded on an agarose gel electrophoresis. The amount of total RNA loaded was assessed by ethidium bromide staining image of 18S and 28S ribosomal RNAs. Northern blot hybridization was done as described previously [1]. Signal intensity was measured with a Bio-Rad GS-800 calibrated densitometer (Bio-Rad Laboratories, Hercules, CA, USA).

2.5. GUS reporter assay

Cell-free extracts were prepared by the method of Tada et al. [15] from mycelia grown under the same conditions as those for RNA preparation. GUS activity was determined spectroscopically by using *p*-nitrophenyl glucuronide as a substrate, according to Jefferson et al. [16].

3. Results and discussion

3.1. Nucleotide sequence analysis of the promoter region of thiA

The nucleotide sequence of *PthiA* (Fig. 1A) contains a putative TATA box (5′-TATTTAA-3′; −528 to −522) and two CT-rich motifs (−494 to −476 and −62 to −42) known to exist in a number of highly expressed genes. Comparison of the *PthiA* sequence with corresponding expressed sequence tag (EST) clones in the *A. oryzae* EST database (http://www.nrib.go.jp/ken/EST/db/index.html) [17] revealed the existence of a putative transcription start site at −475 and two introns (−414 to −359 and −338 to −18) in *PthiA*.

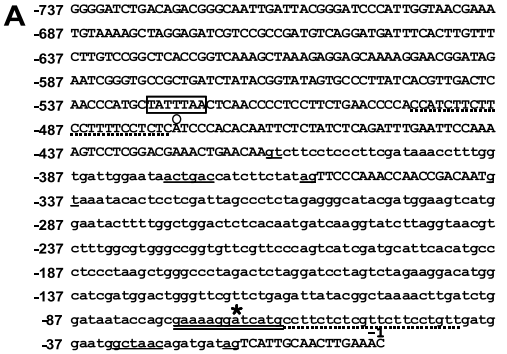

Fig. 1. A: Nucleotide sequence of the 5′-UTR of the *thiA* gene. The transcription start site is indicated by the open circle. The putative TATA box is boxed, and the CT-rich motifs are shown by dashed lines. Lowercase letters represent the introns, and the consensus splicing site sequences of the introns are underlined. Region A is double-underlined, and the mutation point of *ptrA* [1], located in region A, is indicated by an asterisk. B: Alignment of sequences from microbial genes showing partial homology to the *thiA* 5′-UTR. Regions corresponding to −104 to −59 of the *thiA* 5′-UTR are shown. Regions A and B are depicted by double and single lines, respectively.

Table 1
Comparison of GUS activity among deletion mutants

Transformant	TH	ΔA	ΔB
Thiamine −	5531.0 ± 190	1341 ± 55	15.2 ± 10.3
Thiamine +	76.7 ± 5.13	1354 ± 30	5.83 ± 3.79
Ratio (−/+)[a]	72.1	0.99	2.61

TH, ΔA and ΔB were transformants with pNGTH, pNGΔA and pNGΔB, respectively. Cell-free extracts were prepared from mycelia grown in CD medium without thiamine (thiamine −) or containing 10 μM thiamine (thiamine +). Data (nmol *p*-nitrophenol/min/mg protein) are presented as the mean ± S.E.M. of three independent experiments.

[a]Ratio (−/+): the value of GUS activity without thiamine (−) is divided by that with thiamine (+). GUS activity of the transformant with pNG1 (vector control) was not detected (< 0.1).

Results of a preliminary BLAST sequence search [18] indicated that the *thiA*/*ptrA*-encoded protein was highly homologous to Sti35p of *Fusarium solani* [19] and Thi4p of *Saccharomyces cerevisiae* [4]. A motif containing the mutation ($A^{-68}G$) of *ptrA* is highly conserved among their genes (−75 to −63, designated region A). Therefore, to find genes containing region A-like sequences, we performed homology searches of the DNA databases. In addition to the above three genes, *Fusarium oxysporum sti35* [19], *Neurospora crassa nmt-1* (AY007661), and *Campylobacter jejuni thiC* (CJ11168X2) have sequences homologous to *PthiA*. As shown in Fig. 1B, region A is highly conserved among the five genes other than *thiC*. In addition, the nucleotide sequence immediately upstream of region A (−98 to −76, designated region B) of *thiA* is also conserved among them except *THI4*. It is also intriguing that both regions A and B lie within the second intron in the 5′-UTR of *thiA*. These findings prompted us to investigate the function of regions A and B and their involvement in thiamine-dependent gene expression.

3.2. GUS reporter gene analysis of deletion mutants of regions A and B

To elucidate the function of regions A and B in gene expression, we transformed *A. oryzae* niaD300 with pNGΔA and pNGΔB, in which regions A (−75 to −63) and B (−98 to −76) were respectively removed from pNGTH. Southern blot analysis revealed that a single copy of each plasmid integrated into the *niaD* locus of each transformant strain (data not shown). Whereas transformants with pNGTH (which contains both regions A and B; TH, Table 1) showed intensely high GUS activity in the absence of thiamine, exogenous thiamine markedly decreased the GUS activity of the transformant to 1.3% of that in thiamine-deficient medium. Deletion of region A (ΔA, Table 1) resulted in a reduction of GUS activity to 25% of that from TH, and deletion of region B (ΔB) nearly abolished GUS activity (0.2% of that for TH). For both deletion mutants, however, GUS activities did not differ significantly between media containing and lacking thiamine. These results suggest that region B is essential for high-level gene expression, whereas region A is involved in thiamine-dependent repression of *thiA*.

3.3. Northern analysis of deletion mutants of region A or B

We performed Northern analysis of the transformants, us-

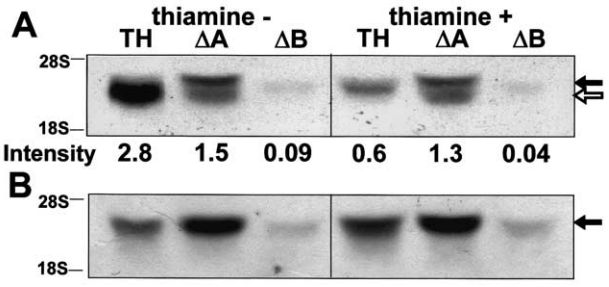

Fig. 2. Northern blot analysis of the *PthiA-uidA* fusion gene in region A and region B deletion mutants. Northern blot analysis was performed by using the *uidA* coding region (A) and the second intron (B) as probes. The blot was over-exposed to make the signals clearer in B. Transformants are the same as shown in Table 1. Total RNAs were prepared from mycelia cultured in CD minimal medium without thiamine (thiamine −) and supplemented with 10 μM thiamine (thiamine +). Signal positions of the mature mRNA and the unspliced pre-mRNA are indicated by an open arrow and closed arrows, respectively. The signal intensity calculated from both RNA species in A is shown below the panel.

ing the *uidA*-encoding region (1.8 kb) as a probe. As expected from the results of the GUS reporter gene analysis, the TH showed the most intense signal for *uidA* in the absence of thiamine (Fig. 2A), and addition of thiamine reduced the intensity of this signal to 20% of that in thiamine-deficient medium. In contrast, the ΔA and ΔB transformants yielded very weak signals for *uidA*, but the signal strength did not differ significantly between thiamine-containing and -deficient media. Intriguingly, transcript levels were not comparable to GUS activities in the presence and absence of thiamine. For instance, whereas the GUS activity of TH in thiamine-deficient medium was >70-fold higher than that in thiamine-containing medium, the transcript level was only five-fold higher. This apparent incongruity suggests that the thiamine-associated regulation of *thiA* gene expression also occurs at the post-transcriptional level. In this context, it should be noted that two bands were detectable for ΔA; the signal position of the lower band (indicated by the open arrow in Fig. 2A) was comparable to that of the intense band observed for TH, and the upper signal (indicated by the closed arrow in Fig. 2A) was apparently absent from TH. The difference in size between the upper and lower bands was estimated to be approximately 300 nucleotides, consistent with the size of the second intron. Thus, we attributed the upper band to pre-mRNA from which the intron(s) in *PthiA* had not been spliced out and the lower band to mature mRNA. In contrast, in the presence of thiamine the signals in TH and ΔB detected by the *uidA* probe ran slightly faster than ΔA pre-mRNA (Figs. 2A and 3A). Furthermore, when hybridized with the intron-specific probe, a much weaker hybridization signal was observed for TH as shown in Fig. 3A,B. These observations suggested that the TH pre-mRNA had been spliced out improperly and thus contain a remnant of the second intron in some pre-mRNA. Partial loss of the intron could explain the shift of band mobility and the weak hybridization signal with the intron probe. Reverse transcription-PCR revealed, in fact, two distinct PCR products: one had the expected size derived from the pre-mRNA, and the other lacked approximately 200 nucleotides from pre-mRNA (data not shown). Therefore, there existed pre-mRNA and improperly spliced mRNA in TH and ΔB in the presence of thiamine.

To ascertain whether the upper (or shifted) band corresponded to pre-mRNA, we performed Northern analysis using the second intron (0.3 kb) as a probe (Fig. 2B). In all transformants, the intron-specific probe hybridized to the upper bands but not to the lower ones. This indicated that the upper and lower bands did, in fact, correspond to the pre-mRNA and spliced transcript, respectively. In TH, the intron-specific probe clearly revealed the upper band in the absence of thiamine. The *thiA* transcript was not spliced completely and a small amount of pre-mRNA remained even in the absence of thiamine. Deletion of regions A resulted in the loss of thiamine-repressed transcription concomitant with a decrease in the splicing efficiency of the transcript. Also in ΔB, transcription was nearly abolished and a very faint upper band was detected by the *uidA* probe (Fig. 2A), which was also demonstrated to be the pre-mRNA (Fig. 2B). This less efficient transcript splicing correlates with a reduction in GUS activity. These observations strongly suggest that exogenous thiamine can modulate *thiA* expression levels through transcript splicing, which may require the regulatory activity of regions A and B.

3.4. *Effect of intron splicing on the thiamine-regulated expression of the GUS reporter gene*

To examine the role of transcript splicing in thiamine-regulated gene expression, we introduced intron splice site mutations by modifying consensus sequences of the second intron in *PthiA*. The consensus 5′ and 3′ splice sites (5′-GT-3′ and 5′-AG-3′) each were altered to 5′-CC-3′ to generate pNG5M and pNG3M, respectively, and both sites were altered to generate pNG5M3M. In addition, the splicing branch point consensus sequence (5′-GCTAAC-3′) was altered to 5′-CCC-CCC-3′ to construct pNGBPM.

In Northern analysis using *uidA* as a probe, all transformants with splice site mutations showed a single upper band (Fig. 3A), which the second-intron-specific probe also detected (Fig. 3B). These findings indicate that these upper bands were pre-mRNAs and that spliced transcripts were rarely formed in transformants in the presence or absence of thiamine. The intensities of the pre-mRNA bands were weak compared with that of the mature mRNA in the control construct TH (Fig. 3A) under non-repressing conditions but were not further reduced when thiamine was added. Transcription and splicing are simultaneously and cooperatively performed on a mRNA factory including RNA polymerase II [20–23]. Therefore block of splicing is presumed to decrease the rate of mRNA synthesis.

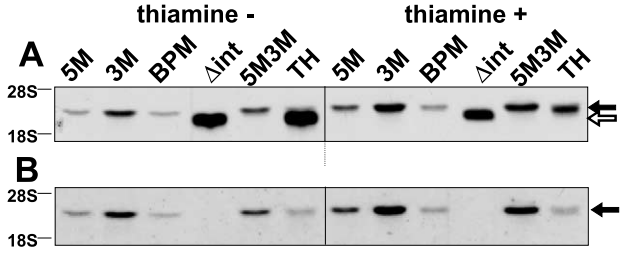

Fig. 3. Northern blot analysis of the *PthiA-uidA* fusion gene in splice site and intronless mutants. Northern blot analysis was performed as described in Fig. 2. Signal positions of the mature mRNA and the unspliced pre-mRNA are indicated by an open arrow and closed arrows, respectively. The probe used was the *uidA* coding region in A and the second intron in B.

Table 2
Comparison of GUS activity of various mutants

Transformant	5M	3M	BPM	Δint	5M3M	TH
Thiamine −	17.2 ± 3.90	56.5 ± 20.3	1.45 ± 0.44	6701 ± 477	8.41 ± 3.66	8436.0 ± 465
Thiamine +	17.1 ± 4.75	4.93 ± 4.48	0.26 ± 0.06	5622 ± 808	9.70 ± 4.65	74.9 ± 6.00
Ratio (−/+)	1.01	11.5	5.49	1.19	0.87	113

5M, 3M, BPM, Δint and 5M3M were transformants with pNG5M, pNG3M, pNGBPM, pNGΔint and pNG5M3M, respectively. The others are the same as shown in Table 1.

Consistent with the Northern analysis, GUS activities in all transformants were reduced to $<1\%$ of that of the non-repressed TH, and their thiamine-repressed expression was nearly abolished (Table 2). Therefore, mutations of consensus sequences of the second intron not only abolished intron splicing but also dramatically downregulated levels of transcription with concomitant loss of thiamine repression. Similar to the results of the deletion experiments, splice site mutations caused greater reduction of GUS activities than expected from the transcription levels, indicating that unspliced transcripts were not translated to functional GUS enzyme. The unspliced pre-mRNA contains many potential translation initiation codons, most of which are followed by translation stop codons prior to the reporter gene AUG. These upstream open reading frames might inhibit initiation or reinitiation of translation at the GUS initiation site.

To further assess the effect of intron splicing on gene expression, we constructed pNGΔint, in which the entire second intron was removed. Transformants with pNGΔint (Δint, Fig. 3) gave a single band corresponding to the mature mRNA, whose intensities in the absence and presence of thiamine were comparable to that of the control construct TH in thiamine-deficient medium. The GUS activities of Δint in both the absence and presence of thiamine were equivalent to the non-repressed level of the TH (Table 2). These findings clearly demonstrate that elimination of the second intron, in which

ID	Accession no. or URL	Organism	Gene	Description
Eco1	NC_000913	*Escherichia coli*	*thiM*	
Aor1	AF217503	*Aspergillus oryzae*	*thiA*	*thi4* homologue, synthesis of thiazole moiety
Aor2	AF217503	*Aspergillus oryzae*	*ptrA*	pyrithiamine resistant mutant allele of *thiA*
Aor3	AB118748	*Aspergillus oryzae*	*nmtA*	*nmt1* homologue
And1	AB111809	*Aspergillus nidulans*	*thiF*	*thi4* homologue
And2	www-genome.wi.mit.edu	*Aspergillus nidulans*		*nmt1* homologue
And3	www-genome.wi.mit.edu	*Aspergillus nidulans*		unknown
Afu1	www.tigr.org	*Aspergillus fumigatus*		*thi4* homologue
Afu2	www.tigr.org	*Aspergillus fumigatus*		*nmt1* homologue
Afu3	www-genome.wi.mit.edu	*Aspergillus fumigatus*		unknown
Fgr1	www-genome.wi.mit.edu	*Fusarium graminearm*		*thi4* homologue
Fgr2	www-genome.wi.mit.edu	*Fusarium graminearm*		*nmt1* homologue
Fox1	AB033416	*Fusarium oxysporum*	*sti35*	*thi4* homologue
Fso1	M33642	*Fusarium solani*	*sti35*	*thi4* homologue
Ncr1	AY007661	*Neurospora crassa*	*nmt-1*	synthesis of pyrimidine moiety
Ncr2	www-genome.wi.mit.edu	*Neurospora crassa*		*thi4* homologue
Mgr1	www-genome.wi.mit.edu	*Magnaprothe grisea*		*thi4* homologue

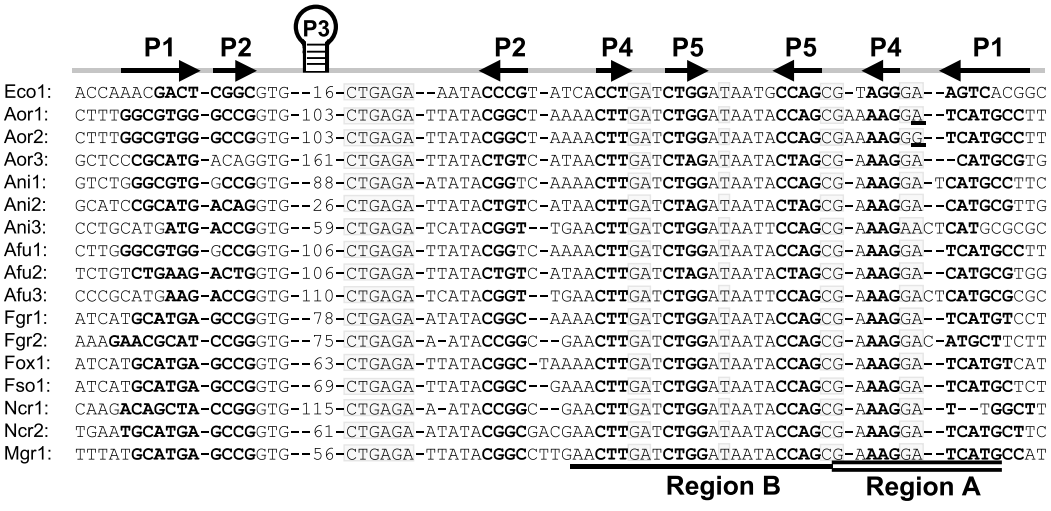

Fig. 4. Alignment of the 5′-UTRs of filamentous fungal genes that have sequences homologous to the bacterial TPP-binding riboswitch. The figure is modified from that of Sudarsan et al. [6] by the addition of sequences (Aor2 and 3, Ani1-3, Afu1-3, Fgr1 and 2, Ncr2, and Mgr1) newly identified in the present study. Arrows and a circle with a ladder above the alignments represent structures of base-paired stems and a stem-loop, respectively. Sequences that form a stem are shown in bold letters and labeled with P1, P2, P4, and P5. Because the P3 sequences are very long, only the numbers of base pairs are indicated. The sequences corresponding to regions A and B are indicated below the alignment. The mutation point in *ptrA* is double-underlined. Sequences highly conserved from prokaryotes to eukaryotes (fungi and plants) are shaded in gray.

regions A and B lie, leads to derepression of the thiamine-regulated *thiA* gene but has almost no effect on the non-repressed expression level. These findings, taken together with the results from the splice site mutations, show that intron splicing is most crucial for thiamine-regulated gene expression.

3.5. *The riboswitch domain in the second intron of the 5′-UTR of* thiA *may affect its thiamine-regulated gene expression*

It has recently been reported that *thiA* contains an RNA element highly homologous to a TPP-binding 'riboswitch' [7]. Riboswitches were initially discovered in the non-coding region of mRNA in prokaryotes, and they serve as metabolite-responsive genetic switches [5,6,24–27]. They interact directly with the target metabolite without conjugation of any protein factors and subsequently lead to modulation of gene expression. Surprisingly, the regions A and B that we focus on constitute a part of the riboswitch-like domain, and the mutation point of *ptrA* is a highly conserved adenosine (Fig. 4, Aor2, underlined) among TPP-binding riboswitches [7]. This mutation causes thiamine-derepressed transcription of the *ptrA* gene [1], suggesting that regions A and B indeed serve as components of the riboswitch-like domain. We must further investigate the effect of the mutation on the TPP-binding ability of the putative riboswitch domain.

We performed sequence homology searches of some genome DNA databases to identify new sequences similar to *PthiA*. The 5′-UTR riboswitch-associated structures occur among filamentous fungi as shown in Fig. 4. These sequences are contained in introns carrying consensus splicing sites and are followed by a putative coding region (data not shown). Comparison of the deduced amino acid sequences showed that except for Ani3 and Afu3, these genes could be classified into two groups: *THI4*-like genes, which participate in the synthesis of the thiazole moiety of thiamine, and *nmt-1* homologues, which are involved in generating the pyrimidine moiety. *N. crassa nmt-1* undergo thiamine-dependent regulation, and Aor3 (*nmtA*; highly homologous to *A. parasiticus nmt-1* [28]) is also repressed strongly by thiamine (H. Shimizu, T. Kubodera and K. Gomi, unpublished results). These observations indicate that pre-mRNA processing (such as intron splicing) directed by a TPP-binding riboswitch is specifically associated with genes involved in thiamine biosynthesis in filamentous fungi.

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