Nascent Peptide-Mediated Translation Elongation Arrest of *Arabidopsis thaliana* CGS1 mRNA Occurs Autonomously

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The *Arabidopsis thaliana* CGS1 gene encodes cystathionine γ-synthase, the first committed enzyme of methionine biosynthesis in higher plants. Expression of CGS1 is feedback-regulated at the step of mRNA degradation in response to S-adenosyl-L-methionine (AdoMet). A short stretch of amino acid sequence, termed the MTO1 region, encoded within the first exon of CGS1 itself acts in cis in the regulation. In vitro analyses using wheat germ extract (WGE) revealed that AdoMet induces temporal translation arrest of CGS1 mRNA prior to mRNA degradation. This translational pausing occurs immediately downstream of the MTO1 region and is mediated by the nascent MTO1 peptide. In order to elucidate further the nature of this unique regulatory mechanism, we have examined whether a non-plant system also contains the post-transcriptional regulation activity. Despite the fact that mammals do not carry cystathionine γ-synthase, AdoMet was able to induce the MTO1 sequence-dependent translation elongation arrest in rabbit reticulocyte lysate (RRL) in a similar manner to that observed in WGE. This result suggests that MTO1 peptide-mediated translation arrest does not require a plant-specific factor and rather most probably occurs via a direct interaction between the nascent MTO1 peptide and the ribosome that has translated it. In contrast, decay intermediates of CGS1 mRNA normally observed upon induction of CGS1 mRNA decay in plant systems were not detected in RRL, raising the possibility that CGS1 mRNA degradation involves a plant-specific mechanism.

Keywords: S-Adenosyl-l-methionine — *Arabidopsis thaliana* — mRNA stability — Translation elongation arrest.

Abbreviations: AdoMet, S-adenosyl-l-methionine; CGS, cystathionine γ-synthase; CTAB, cetyltrimethylammonium bromide; GST, glutathione S-transferase; LUC, firefly luciferase; ORF, open reading frame; RLUC, sea pansy luciferase; RRL, rabbit reticulocyte lysate; uORF, upstream ORF; WGE, wheat germ extract.

Introduction

Methionine is an essential amino acid for non-ruminant animals, which do not have enzymes for methionine biosynthesis. The *Arabidopsis thaliana* CGS1 gene (gene ID At3g01120) encodes cystathionine γ-synthase (CGS; EC 2.5.1.48) that catalyzes the first committed step of methionine biosynthesis in higher plants (Kim and Leustek 1996, Kim et al. 1999). CGS converts O-phosphohomoserine and cysteine into cystathionine, and this step constitutes the key regulatory step in the methionine biosynthetic pathway. However, unlike many of the key steps in biosynthetic pathways, CGS is not an allosteric enzyme (Thompson et al. 1982). Expression of the CGS1 gene is feedback-regulated post-transcriptionally at the step of mRNA degradation in response to S-adenosyl-l-methionine (AdoMet), a direct metabolite of methionine (Chiba et al. 1999, Chiba et al. 2003). CGS1 mRNA degradation was accelerated when *Arabidopsis* calli were fed with methionine to increase the AdoMet level in vivo. In addition, 5′-truncated *CGS1* RNA species representing a decay intermediate were observed upon the induction of *CGS1* mRNA decay (Chiba et al. 1999, Lambein et al. 2003). A short stretch of amino acid sequence encoded by the first exon of CGS1, termed the MTO1 region, is involved in this regulation by acting in cis (Chiba et al. 1999, Suzuki et al. 2001, Ominato et al. 2002). The *mto1* mutants of *Arabidopsis*, which carry single amino acid sequence alterations within the MTO1 region, are defective in this regulation and overaccumulate soluble methionine (Chiba et al. 1999, Ominato et al. 2002).

The post-transcriptional regulation of *CGS1* was recapitulated in the plant in vitro translation system of wheat germ extract (WGE) (Chiba et al. 2003). When WGE was programmed with RNA carrying *CGS1* exon 1 fused to a reporter gene, AdoMet induced down-regulation of reporter activity and accumulation of 5′-truncated *CGS1*...
RNA species that had the same 5' ends as those detected in vivo. In addition, we found that translational elongation is temporarily arrested in response to AdoMet prior to production of the 5'-truncated RNA species (Onouchi et al. 2005). This translational pausing occurs at the Ser94 codon located immediately downstream of the MTO1 region. It was also found that the amino acid sequence, rather than the nucleotide sequence, of the MTO1 region is responsible for the translation elongation arrest. Together with the cis-acting nature of the MTO1 region, these observations led us to propose a model in which the amino acid sequence encoded by the MTO1 region in nascent CGS1 polypeptide acts within the ribosome that has just translated it to cause translational pausing in response to AdoMet, which then triggers mRNA degradation (Onouchi et al. 2005).

It remained to be determined whether factors other than the MTO1 region amino acid sequence are involved in the post-transcriptional regulation of CGSI. In the present study, we investigated whether the regulation can be reproduced in the non-plant in vitro translation system of rabbit reticulocyte lysate (RRL). If plant-specific factors are required, the regulation would not be recapitulated in RRL. Since an amino acid sequence similar to the MTO1 peptide cannot be found in the public database other than in plant CGSs (Ominato et al. 2002), it is unlikely that mammalian cells have a factor that can substitute for the function of possible plant-specific factors. We show here that when RRL was programmed with an RNA harboring CGSI exon 1, translation elongation arrest was induced by AdoMet in a similar manner to that observed in WGE, although the RNA decay intermediates were not observed. These results suggest that no plant-specific factor other than the MTO1 peptide is required to mediate the AdoMet-induced translation elongation arrest, whereas additional plant-specific factors may be required for the coupled mRNA degradation.

**Results**

**MTO1 peptide-mediated downregulation of gene expression is induced in RRL.**

In previous studies using WGE, MTO1 peptide-mediated regulation was investigated using Ex1:Luc RNAs that contain CGSI exon 1 fused in-frame to a Luc gene encoding firefly luciferase (LUC). AdoMet was shown to induce down-regulation of an Ex1:Luc RNA containing wild-type CGSI exon 1 [Ex1(WT):Luc], whereas no such regulation was observed with a mto1-1 mutant version [Ex1(mto1-1):Luc] that acted as a negative control (Onouchi et al. 2005). The mto1-1 mutation is a Gly84 to serine substitution within the MTO1 region and abolishes the AdoMet-induced regulation in plants as well as in WGE (Chiba et al. 1999, Chiba et al. 2003). To determine whether

![MTO1 peptide-mediated down-regulation of LUC reporter activity in RRL](image)

**Fig. 1** MTO1 peptide-mediated down-regulation of LUC reporter activity in RRL. (A) Schematic representation of Ex1(WT):Luc and Ex1(mto1-1):Luc RNA. (B) In vitro transcripts shown in (A) were translated in RRL for 60 min in the presence or absence of 1 mM AdoMet. LUC activity was normalized with control RLUC activity from co-translated RLUC RNA, and reporter activity relative to samples without AdoMet (+AdoMet/–AdoMet) was calculated. Averages ± SD of triplicate experiments are shown.

CGSI exon 1 is also able to mediate down-regulation of gene expression in a non-plant system, these RNAs were used in the current study to monitor the effect of AdoMet on reporter gene expression in RRL. All RNA templates used for in vitro translation assays were prepared from the DNA constructs by in vitro transcription, were capped and had an A30 tail (see Materials and Methods). Ex1(WT):Luc RNA and Ex1(mto1-1):Luc RNA (Fig. 1A) were translated in RRL in both the presence and absence of AdoMet. RLUC RNA encoding sea pansy luciferase (RLUC) was co-translated as an internal control. Following a 60 min incubation period of the translation reaction, LUC and RLUC reporter activities were measured and LUC activity was normalized against RLUC activity. As shown in Fig. 1B, LUC activity was repressed when Ex1(WT):Luc RNA was translated in the presence of AdoMet. In contrast, AdoMet treatment did not influence reporter activity when Ex1(mto1-1):Luc RNA was used. This result indicates that AdoMet can induce MTO1 peptide-mediated down-regulation of reporter gene expression in the non-plant RRL system.

**MTO1 peptide-mediated translation elongation arrest is induced in RRL.**

In order to clarify which steps of the CGSI post-transcriptional regulation are recapitulated in RRL, we first
examined whether the translation arrest is induced in RRL. We have previously shown that when RNA harboring a glutathione S-transferase (GST)-tagged Ex1(WT):Luc construct \([\text{GST:Ex1(WT):Luc}]\) was translated in WGE supplemented with AdoMet, accumulation of an approximately 55 kDa peptidyl-tRNA was observed due to the translation elongation arrest that occurred immediately downstream of the \(\text{MTO1} \) region (Onouchi et al. 2005). We therefore tested for the presence of AdoMet-induced translation arrest products in RRL using the same GST-tagged constructs.

RNAs transcribed from the wild-type \([\text{GST:Ex1(WT):Luc}]\) and mutant \([\text{GST:Ex1(mto1–1):Luc}]\) (Fig. 2A) were translated in RRL in the presence or absence of AdoMet. Translation products were then analyzed by immunoblot analysis using anti-GST antibody. The filled arrowhead indicates the position of AdoMet-dependent peptidyl-tRNA (55 kDa), and the open arrowhead indicates the position of the full-length translation product (110 kDa). The positions of protein size markers are indicated. Representative results of triplicate experiments are shown. (B) Immunoblot analysis of translation products. In vitro transcripts shown in (A) were translated in RRL for 0, 30 or 60 min in the presence (+) or absence (−) of 1 mM AdoMet, as indicated. Translation products were analyzed by immunoblot analysis using an anti-GST antibody. The filled arrowhead indicates the position of AdoMet-dependent peptidyl-tRNA (55 kDa), and the open arrowhead indicates the position of the full-length translation product (110 kDa). The positions of protein size markers are indicated. Representative results of triplicate experiments are shown. (C) Immunoblot analysis of translation products after CTAB precipitation and/or RNase A treatment. In vitro transcripts shown in (A) were translated in RRL for 30 min in the presence of AdoMet (lane 4). In lanes 1–3, translation products were precipitated with CTAB, and supernatants (sup; lane 3) and precipitates (ppt; lanes 1 and 2) were analyzed. In lane 1, precipitates were treated with RNase A before analysis. In lane 5, the in vitro translation reaction mixture was directly treated with RNase A. Open, filled and gray arrowheads indicate the positions of full-length translation product (110 kDa), AdoMet-dependent peptidyl-tRNA (55 kDa) and the AdoMet-dependent band shifted by RNase A treatment (35 kDa), respectively. The positions of protein size markers are indicated. A representative result of triplicate experiments is shown.

Fig. 2 Translation elongation arrest in RRL. (A) Schematic representation of \([\text{GST:Ex1(WT):Luc}]\) and \([\text{GST:Ex1(mto1–1):Luc}]\) RNA. (B) Immunoblot analysis of translation products. In vitro transcripts shown in (A) were translated in RRL for 0, 30 or 60 min in the presence (+) or absence (−) of 1 mM AdoMet, as indicated. Translation products were analyzed by immunoblot analysis using an anti-GST antibody. The filled arrowhead indicates the position of AdoMet-dependent peptidyl-tRNA (55 kDa), and the open arrowhead indicates the position of the full-length translation product (110 kDa). The positions of protein size markers are indicated. Representative results of triplicate experiments are shown. (C) Immunoblot analysis of translation products after CTAB precipitation and/or RNase A treatment. In vitro transcripts shown in (A) were translated in RRL for 30 min in the presence of AdoMet (lane 4). In lanes 1–3, translation products were precipitated with CTAB, and supernatants (sup; lane 3) and precipitates (ppt; lanes 1 and 2) were analyzed. In lane 1, precipitates were treated with RNase A before analysis. In lane 5, the in vitro translation reaction mixture was directly treated with RNase A. Open, filled and gray arrowheads indicate the positions of full-length translation product (110 kDa), AdoMet-dependent peptidyl-tRNA (55 kDa) and the AdoMet-dependent band shifted by RNase A treatment (35 kDa), respectively. The positions of protein size markers are indicated. A representative result of triplicate experiments is shown.

In addition to its 55 kDa size, the AdoMet-induced translation arrest product in WGE is defined by three additional specific characteristics: (i) the product contains a tRNA; (ii) the translation elongation arrest is temporal; and (iii) translation arrest occurs at Ser94 (Onouchi et al. 2005). If the 55 kDa band observed in the RRL assay is identical to that in WGE, and the same mechanism is indeed responsible for the translation arrest in both RRL and WGE, the 55 kDa product induced by AdoMet in RRL would also have to meet these three criteria.

As a test for the presence of tRNA, the 55 kDa band induced in RRL was treated with RNase A (Fig. 2C). This treatment resulted in a shift of the band to approximately 35 kDa as was the case in WGE (Onouchi et al. 2005). In addition, the 55 kDa band was precipitable with cetyltrimethylammonium bromide (CTAB), which precipitates nucleic acids (Fig. 2C). These results corroborate our notion that this partial translation product is a peptidyl-tRNA.
The temporal status of the translation elongation arrest in RRL was next examined in a pulse–chase experiment using \([^{35}S]\)methionine and GST:Ex1 RNA without the LUC reporter (Fig. 3A). The GST coding region carries nine methionine codons, while the CGS1 exon 1 coding region has only the first methionine codon (Onouchi et al. 2005). At 5 min after the start of the translation reaction, edeine, an inhibitor of translation initiation (Dinos et al. 2004), was added to inhibit new rounds of translation and the translation products were then monitored between 10 and 120 min. As shown in Fig. 3B, the presence of AdoMet resulted in an increase in the 55 kDa peptidyl-tRNA band up to 15 min, after which it declined to below detection levels by 120 min. This decrease in the 55 kDa band matched with an increase in intensity of the full-length 45 kDa peptide band. Although the intensity of the 55 kDa band reached a maximum at an earlier time point than that previously observed in WGE (30 min), the rate of decrease and thus the temporal status of translation elongation arrest was similar to that in WGE.

The exact position of translation arrest in RRL was next determined by introducing stop codon substitutions around the MTO1 region within GST:Ex1:Luc constructs. A stop codon placed at or upstream of the arrest site should abolish accumulation of the partial translation product, whereas a stop codon placed downstream of the arrest site should not affect the translation arrest. For clarity, the positions of the amino acid residues are numbered from the first methionine of CGS. As shown in Fig. 4, when the amber stop codon (UAG) was introduced at residues Ile88 (I88UAG), Lys92 (K92UAG), Trp93 (W93UAG) or Ser94 (S94UAG), the AdoMet-dependent 55 kDa band was not detected. In contrast, UAG substitutions at Asn96 (N96UAG) and Pro97 (P97UAG) did not affect the induction of the 55 kDa band detected in the wild-type construct. These results indicate that translation arrest occurs at Ser94 in RRL, as is the case in WGE.

The above results confirm that the 55 kDa AdoMet-induced translation arrest product detected in RRL is indeed the same as that observed in WGE, indicating that MTO1 peptide-mediated translation elongation arrest is induced in RRL in a similar manner to that in WGE.

mRNA degradation is not coupled with the MTO1 peptide-mediated translation arrest in RRL

In WGE, translation elongation arrest mediated by MTO1 peptide is followed by production of short RNA species that lack the 5’ region and are thought to be decay intermediates (Chiba et al. 2003, Onouchi et al. 2005). We next examined whether production of such 5’-truncated RNA species is also recapitulated in RRL.

To detect 5’-truncated RNA species in RRL, primer extension analysis was carried out. In WGE, primer extension analysis detected a ladder of 5’ ends of the 5’-truncated RNA species upstream of the Ser94 codon, the translation arrest site. These 5’ end points are thought to be produced by RNA degradation events that occur near the 5’ edge of the stalled ribosome as well as those that are stacked behind the initial stalled ribosome (Haraguchi et al. 2008). GST:Ex1(WT):Luc RNA was translated in the presence and absence of AdoMet in both WGE and RRL, after which poly(A) RNA was extracted and analyzed by primer extension analysis. The GST tag sequence is not necessary for detection of the RNA degradation intermediates; however, the 5’-truncated RNA ladder is more clearly detected with GST:Ex1(WT):Luc RNA in WGE.
they would probably be general factors that are not additional factors conserved between plants and animals, arrested. Although we cannot rule out the involvement of required for the AdoMet-induced translation elongation specific factor other than the to the case in WGE. These results suggest that no plant-specific trans-acting factor is not required for MTO1 peptide-mediated translation elongation arrest of CGS1 mRNA

Our previous studies using WGE revealed that translation of CGS1 mRNA is temporally arrested in response to AdoMet prior to mRNA degradation, and that the MTO1 region amino acid sequence is responsible for the translation elongation arrest (Onouchi et al. 2005). Here, we demonstrated that AdoMet induces MTO1 peptide-mediated translational pausing in the mammalian RRL system in a similar manner to that in WGE. When RNA carrying wild-type CGS1 exon 1 was translated in RRL supplemented with AdoMet, translation paused at exactly the same position as in WGE, and resumed similarly to the case in WGE. These results suggest that no plant-specific factor other than the cis-acting MTO1 peptide is required for the AdoMet-induced translation elongation arrest. Although we cannot rule out the involvement of additional factors conserved between plants and animals, they would probably be general factors that are not specifically involved in the CGS1 regulation, because mammals do not have CGS, and an amino acid sequence similar to the MTO1 region cannot be found in the public database other than in plant CGSs (Ominato et al. 2002), and therefore it is unlikely that CGS1-specific regulation is conserved in mammals. A plausible model is that both AdoMet and the MTO1 peptide interact directly with the translation elongation complex to cause the translation arrest. Translation arrest occurs at the Ser94 codon located immediately downstream of the MTO1 region, suggesting that the nascent MTO1 peptide resides in the stalled ribosome when translation is arrested (Onouchi et al. 2005). This supports the idea that the nascent MTO1 peptide interacts directly with the ribosome that has translated it to mediate the translation arrest.

One possible mode of direct action of AdoMet on the translation elongation complex could be methylation of a translational machinery component using AdoMet as a methyl donor. This modification could then make the ribosome sensitive to the inhibitory effect of the MTO1 peptide, resulting in translation elongation arrest. An observation that argues against this possibility is that S-adenosyl-L-homocysteine, a competitive inhibitor of the AdoMet-dependent methylation reaction, did not exhibit any competitive effect with AdoMet on the MTO1 peptide-mediated regulation in WGE (Chiba et al. 2003). Another possibility is that, without the methyltransfer reaction, direct binding of AdoMet to the translation elongation complex including the nascent peptide causes translation arrest by modulating sensitivity of the ribosome to the MTO1 peptide or acting cooperatively with the MTO1 peptide within the translating ribosome.

Several examples of nascent peptide-mediated ribosomal regulation have been reported (for reviews, see Lovett and Rogers 1996, Tenson and Ehrenberg 2002).
Interestingly, nascent peptides encoded by the *Escherichia coli* secM and *tnaC* genes have been shown to interact directly with components of the ribosomal exit tunnel to cause translation arrest (Nakatogawa and Ito 2002, Cruz-Vera et al. 2005). Furthermore, it has been suggested that a small inducer molecule, tryptophan, binds directly to a ribosome to induce TnaC peptide-mediated ribosomal inhibition at the step of translation termination by interfering with the activity of the peptidyltransferase center (Gong and Yanofsky 2002, Cruz-Vera et al. 2006, Cruz-Vera et al. 2007).

Apart from the *CGS1* gene, eukaryotic examples of nascent peptide-mediated ribosomal regulation involve peptides encoded by upstream open reading frames (uORFs) that cause ribosome stalling at the stop codon of the uORF (Cao and Geballe 1996, Wang and Sachs 1997, Wang et al. 1999, Law et al. 2001, for a review see Morris and Geballe 2000). In these cases, the stalled ribosome blocks translation initiation of a downstream ORF. Among them, uORF peptide-mediated regulation of *Neurospora crassa* arg-2 (and its *Saccharomyces cerevisiae* ortholog, CPA1) and mammalian AdoMet decarboxylase is induced by arginine and polyamine, respectively, which are the products of metabolic pathways in which these genes are involved (Wang et al. 1999, Law et al. 2001, Mize and Morris 2001). In these systems, these low molecular weight compounds can induce the regulation even when the uORFs were introduced into heterologous systems, namely *N. crassa* arg-2 and *S. cerevisiae* CPA1 genes in WGE (Wang et al. 1999), the arg-2 gene in RRL (Fang et al. 2004) and the mammalian AdoMet decarboxylase gene in *S. cerevisiae* (Mize and Morris 2001), in which regulation of the corresponding gene is not conserved. Thus, similarly to the *CGS1* mRNA translation arrest, specific factors do not appear to be required in these systems. The unique feature of the *CGS1* regulation is that the nascent regulatory peptide is encoded by the same ORF that codes for the CGS enzyme, although the arg-2 uORF peptide was shown to be capable of inducing translation elongation arrest when the uORF was fused in-frame with another ORF (Fang et al. 2004). It remains to be elucidated whether these uORF systems share a common mechanism with the *CGS1* regulation. The data presented in the present study not only add to the repertoire of heterologous conservation of translation arrest mechanisms, but also provide means by which we may explore the complex nature of the *CGS1* regulation system.

**Possible uncoupling of *CGS1* mRNA degradation from translation arrest in RRL**

We have previously shown that nascent MTO1 peptide-mediated translation arrest is coupled with production of *CGS1* mRNA decay intermediates in WGE (Onouchi et al. 2005). Strong correlation between these two events was observed by mutational analysis of the MTO1 region amino acid sequence, suggesting that the translation arrest triggers *CGS1* mRNA degradation in plant cells (Onouchi et al. 2005). In contrast, such decay intermediates were not detected in RRL even under
conditions that resulted in translation arrest. Although a possibility remains that the 5'-truncated RNA species, whose half-life in \textit{Arabidopsis} calli is about 30 min (Lambein et al. 2003), is very unstable in RRL, the results obtained here suggest that a plant-specific mechanism may be required for \textit{CGS}1 mRNA degradation to be triggered by the translation arrest. Alternatively, mammalian cells could contain a functionally equivalent mechanism that is inactive in a cell-free condition or in specialized cells such as reticulocytes.

\section*{Materials and Methods}

\subsection*{Chemicals}
AdoMet was purchased from Sigma-Aldrich (St Louis, MO, USA). Edeine sulfate was a gift from National Cancer Institute (Bethesda, MD, USA). Other chemicals used are described in Chiba et al. (2003).

\subsection*{Plasmids}
Plasmids pMI21(WT) and pMI21(mto1-1), which carry the \textit{Ex1(WT)}:Luc and \textit{Ex1(mto1-1)}:Luc DNA in the pSP64 Poly(A) vector (Promega, Madison, WI, USA), respectively, were described previously (Chiba et al. 2003). Plasmids pYN10(WT), pYN10(mto1-1), pMN11(WT) and pMN11(mto1-1), which carry the GST:Ex1(WT):Luc, GST:Ex1(mto1-1):Luc, GST:Ex1(WT)/ and GST:Ex1(mto1-1) DNA in the pSP64 Poly(A) vector (Promega), respectively, were described previously (Onouchi et al. 2005). Plasmids carrying \textit{amber} stop codon (TAG) substitutions in the GST:Ex1(WT):Luc construct were also described previously (Onouchi et al. 2005).

\subsection*{In vitro transcription}
DNA templates in pSP64 poly(A) vector (Promega) were linearized with \textit{EcoRI}, purified, and transcribed in vitro in the presence of a cap analog \textit{m}^3G\textit{m}\textit{p}\textit{p}\textit{p}\textit{p}\textit{S}GTP as described (Chiba et al. 2003).

\subsection*{In vitro translation}
In vitro translation reactions in RRL were carried out using the Flexible Rabbit Reticulocyte Lysate System (Promega). The standard reaction mixture contained 33 \text{mM} of RRL, 3.5 \text{mM} of 1 \text{M KOAc}, 1.0 \mu\text{M} of 1 \text{mM} amino acid mixture lacking methionine (Promega), 1.0 \mu\text{M} of 5 \mu\text{M} l-methionine and 50 \text{U} of recombinant RNasin ribonuclease inhibitor (Promega), for a total volume of 50 \text{\mu}l. MgOAc was added to adjust the final concentration of Mg\textsuperscript{2+} to 1.2 \text{mM}. AdoMet was added at 1 \text{mM} final concentration when its effect was analyzed. In vitro synthesized RNA was added to the reaction mixture at a final concentration of 2 \text{fmol} \mu\text{M} \text{for primer extension experiments, and 50 fmol} \mu\text{M} \text{for immunoblot and pulse-chase experiments. For the LUC assay, 2 fmol} \mu\text{M of} \textit{Ex1:Luc} RNA and 1 fmol} \mu\text{M of} \textit{RLuc} RNA were used. All reaction mixtures were incubated at 30 \text{C}. LUC and RLUC activities were assayed as described (Chiba et al. 2003). For RNase A treatment, RNase A was added at a final concentration of 0.5 \mu\text{g} \mu\text{M}, and the reaction mixtures were incubated for 15 min at 37 \text{C}. CTAB precipitation was performed as described (Nakatogawa and Ito 2001).

In vitro translation reactions using WGE (Promega) were carried out as described (Chiba et al. 2003).
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