

S-adenosyl-L-methionine is an effector in the posttranscriptional autoregulation of the cystathionine γ -synthase gene in *Arabidopsis*

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Cystathionine γ -synthase, the first committed enzyme of methionine biosynthesis in higher plants, is encoded by the *CGS1* gene in *Arabidopsis thaliana*. We have shown previously that the stability of the *CGS1* mRNA is negatively regulated in response to methionine application [Chiba, Y., Ishikawa, M., Kijima, F., Tyson, R. H., Kim, J., Yamamoto, A., Nambara, E., Leustek, T., Wallsgrave, R. M. & Naito, S. (1999) *Science* 286, 1371–1374]. To determine whether methionine itself is the effector of the *CGS1* exon 1-mediated posttranscriptional regulation, we carried out transfection experiments. The results suggested that, rather than methionine, S-adenosyl-L-methionine (AdoMet), or one of its metabolites, acts as the effector of this regulation. To further identify the actual effector, we exploited the wheat germ *in vitro* translation system. The effects of various metabolites and analogs of AdoMet were tested by using RNA carrying a *CGS1* exon 1-reporter fusion. These tests identified AdoMet as the effector of this regulation. S-adenosyl-L-ethionine, an analog of AdoMet, also had effector activity. *A. thaliana mto1* mutants, which are deficient in this regulation, showed a much reduced response to AdoMet *in vitro*, with a leaky allele showing a less reduced response. RNA translated *in vitro* in the presence of AdoMet contained a 5'-truncated RNA species, similar to the one that we previously suggested was an *in vivo* degradation intermediate of *CGS1* mRNA. Together, the results show that the basic reactions of *CGS1* exon 1-mediated posttranscriptional regulation occur in the wheat germ *in vitro* translation system, and that AdoMet acts as the effector.

Cystathionine γ -synthase (CGS; EC 4.2.99.9) catalyzes the first committed step of methionine biosynthesis in higher plants (1) (Fig. 1), which is considered to be the key regulatory step in methionine biosynthesis (2–5). Unlike many of the key-step enzymes in metabolic pathways, CGS is not an allosteric enzyme (2). CGS is encoded in *Arabidopsis thaliana* by the *CGS1* gene (gene ID At3g01120, GenBank accession no. AB010888) (6, 7). We have previously shown (8) that *CGS1* expression involves feedback regulation at the level of mRNA stability in response to methionine application *in vivo*. *A. thaliana mto1* mutants are deficient in this feedback regulation, and overaccumulate *CGS1* mRNA, CGS protein, and soluble methionine. Seven independently isolated *mto1* mutants were found to carry single-base changes within the first exon of *CGS1*, giving rise to amino acid sequence changes (8, 9).

Transient and transgenic expression experiments using *CGS1* exon 1-reporter fusions (8, 10) showed that the exon 1 coding sequence of *CGS1* is necessary and sufficient for its posttranscriptional regulation, in response to exogenous application of methionine. *In vitro* mutagenesis of *CGS1* exon 1 revealed that it is its amino acid sequence that has a role in this regulation. We have identified a stretch of 11–13 amino acid residues, termed the MTO1 region, located \approx 80 residues from the N terminus of CGS, and covering the *mto1* mutation sites, which is important for this regulation. The MTO1 sequence is highly conserved among the CGS proteins of higher plants, but is not found elsewhere in the public nucleotide and protein databases (9).

Despite the fact that the amino acid sequence of exon 1 is crucial, the exon 1 sequence has been shown to act in cis. To account for this action, we have presented a model in which the regulation occurs during the translation of *CGS1* mRNA (8, 10).

In the 1980s, it was shown that CGS enzyme activity in the aquatic plant *Lemna paucicostata* is repressed when the plant is grown in the presence of methionine, and it has been believed that methionine feedback-regulates its own *de novo* biosynthesis (2, 11), although the mechanism of this regulation was unknown. We have shown that expression of *CGS1* is regulated at the level of mRNA stability (8), but it still remained to be determined whether methionine itself, or one of its metabolites, is the actual effector of this regulation. Establishment of an *in vitro* system would not only be useful in identifying the effector, but would also greatly facilitate the elucidation of the molecular mechanisms of *CGS1* regulation. According to our model that the regulation occurs during translation, the regulation could be reproduced in an *in vitro* translation system. We show here that the posttranscriptional regulation mediated by exon 1 of *CGS1* occurs in the wheat germ *in vitro* translation system, and that S-adenosyl-L-methionine (AdoMet) acts as the effector.

Materials and Methods

Chemicals. The chemicals used to test the effector activity in the regulation of *CGS1* expression were as follows: L-methionine (catalog no. 133-01602, Wako Chemicals, Osaka), L-homocysteine (catalog no. 085-07591, Wako Chemicals), 1-aminocyclopropane-1-carboxylic acid (catalog no. 010-17711, Wako Chemicals), AdoMet (catalog no. A-4377, Sigma-Aldrich, St. Louis), S-adenosyl-L-homocysteine (AdoHcy; catalog no. A-9384, Sigma-Aldrich), 5'-deoxy-5'-(methylthio)adenosine (catalog no. 31122, Fluka, Buchs, SG, Switzerland), S-adenosyl-L-ethionine (AdoEtn; catalog no. A-2758, Sigma-Aldrich), adenosyl-L-ornithine (AdoOrn; catalog no. 56705, Calbiochem), S-methyl-L-methionine (SMM; catalog no. 155398, ICN), S-adenosyl-L-propionine (AdoPpn; synthesized by Eburon Organics, Rijkvorsel, Belgium), and decarboxylated AdoMet (dcAdoMet; a gift of A. Shirahata, Josai University, Saitama, Japan; ref. 12).

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Abbreviations: CGS, cystathionine γ -synthase; AdoMet, S-adenosyl-L-methionine; AdoHcy, S-adenosyl-L-homocysteine; dcAdoMet, decarboxylated AdoMet; AdoEtn, S-adenosyl-L-ethionine; SMM, S-methyl-L-methionine; AdoPpn, S-adenosyl-L-propionine; AdoOrn, adenosyl-L-ornithine; Col-0, Columbia WT; Ws, Wassilewskija WT; LUC, *Photinus pyralis* luciferase; RLuc, *Renilla reniformis* luciferase; GUS, β -glucuronidase.

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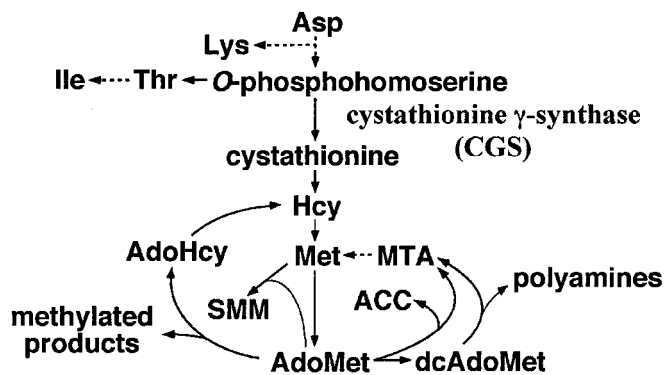


Fig. 1. Schematic representation of the biosynthetic pathway of methionine and the metabolism of AdoMet. Dashed arrows represent multiple reactions. Hcy, L-homocysteine; AdoHcy, S-adenosyl-Hcy; MTA, 5'-deoxy-5'-(methylthio)-adenosine; ACC, 1-aminocyclopropane-1-carboxylic acid; SMM, S-methyl-L-methionine; dcAdoMet, decarboxylated AdoMet.

Genetic Materials. The *A. thaliana* (L.) Heynh. mutants *mtol-1*, *mtol-2*, *mtol-3*, *mtol-4* (8, 9), and *mtol-3-1* (13) have been described elsewhere. *mtol-1* and *mtol-3-1* mutants and their WT strains, ecotypes Columbia (Col-0) and Wassilewskija (Ws), respectively, were used in the transfection experiments. The *mtol-3-1* mutant and Ws were obtained from M. C. Tarczynski (Pioneer Hi-Bred International, Johnston, IA).

Transfection Experiments. The tester plasmids pMI4(WT) and pMI4(*mtol-1*), and the control plasmid 221-LUC+ have been described (9, 14). Transfection of *A. thaliana* protoplasts by electroporation, treatment of the electroporated protoplasts, and reporter assays were carried out as described (9).

Construction of Plasmid DNA for *in Vitro* Transcription. pMI21(WT) plasmid carries the exon 1 coding region of WT *CGS1* joined in-frame with the *luc+* gene, which codes for the firefly (*Photinus pyralis*) luciferase (LUC), in the pSP64 Poly(A) vector (Promega). The exon 1 coding region, carrying *XbaI* and *BamHI* recognition sequences at its 5' and 3' ends, respectively, was prepared as described (9). The *luc+* coding region was excised from the pSP64 *luc+* plasmid (Promega) by digestion with *HindIII* and *XbaI*. The linker 5'-TCTAGGCGGAGGTGG-GAGCTC-3' was ligated to the 3' end of the *luc+* DNA, destroying the *XbaI* recognition sequence. The exon 1 region and the *luc+* DNA were joined with the linker 5'-GGATCCGGCG-GTGGAAAGCTT-3', and were inserted between the *XbaI* and *SacI* sites of the pSP64 Poly(A) vector. *mtol-1*, *mtol-2*, *mtol-3*, and *mtol-4* mutant versions of the above plasmid were also constructed, and are referred to as pMI21(*mtol-1*), pMI21(*mtol-2*), pMI21(*mtol-3*), and pMI21(*mtol-4*). The pMI27 plasmid carries the sea pansy (*Renilla reniformis*) luciferase (RLUC) reporter gene, termed *Rluc*, in the pSP64 Poly(A) vector. The *Rluc* coding sequence was excised by digestion of the pRL-null vector (Promega) with *NheI* and *XbaI*, and was cloned into the pSP64 Poly(A) vector at its *XbaI* site.

***In Vitro* Transcription.** *In vitro* transcription of *EcoRI*-linearized plasmid DNAs by SP6 polymerase was carried out by using the AmpliCap SP6 high-yield message maker kit (Epicentre Technologies, Madison, WI) in the presence of the cap analog m⁷G[5']ppp[5']GTP (Epicentre Technologies). After *in vitro* transcription, template DNA was digested with RNase-free DNase I, and the RNA was purified by using an RNeasy minikit (Qiagen, Valencia, CA), and was poly(A)-selected by using a GenElute mRNA miniprep kit (Sigma-Aldrich). The capped RNA transcribed from pMI21(WT) will be referred to as

Ex1(WT):Luc RNA, with the allele names used to indicate the *mtol* mutant versions, and that which is transcribed from pMI27 will be referred to as *Rluc* RNA.

***In Vitro* Translation in Wheat Germ Extract.** The standard reaction mixture contained 25 μ l of wheat germ extract (Promega), 4 μ l of 1 mM amino acid mixture lacking methionine (Promega), 1 μ l of 5 μ M L-methionine, 50 units of RNasin (Promega), 100 fmol of *Ex1(WT):Luc* RNA or one of its *mtol* mutant derivatives, and 50 fmol of *Rluc* RNA, for a total volume of 50 μ l. All reactions were carried out at 25°C for 120 min. LUC and RLUC activities were assayed by using a dual-luciferase reporter assay kit (Promega) and a Lumat LC9507 luminometer (Berthold, Nashua, NH). RNA extraction and Northern analysis were carried out as described (10) after poly(A) selection. The LUC probe was a 1.7-kb *HindIII-SacI* fragment of pMI21(WT). The 5' probe that covers nucleotides 10–213 relative to the first ATG of *CGS1* was prepared by PCR amplification of pMI21(WT) DNA, using the primers 5'-TCATCATTCCAGTGCCTAC-CATCTTC-3' and 5'-CTGACGGACGAAATTAGGAG-GAAATC-3'. The 3' probe that covers nucleotides 1448–1650 relative to the first ATG of *luc+* was prepared by PCR amplification of pMI21(WT) DNA, using the primers 5'-GCCGTT-GTTGTTTTGGAGCAC-3' and 5'-ACGGCGATCTTTC-CGCCCTTC-3'. The *Rluc* gene probe was a 1.0-kb *NaeI-XbaI* fragment of pMI27. ³²P-labeled probes were prepared by using a Megaprime DNA labeling system (Amersham Pharmacia Biosciences). Hybridization signals were detected by using a BAS 1000 bioimage analyzer (Fuji).

Primer Extension Studies. Poly(A) RNA from an *in vitro* translation reaction lacking *Rluc* RNA was denatured for 5 min at 67°C, and was immediately placed on ice. The primer extension reaction was carried out by using Thermoscript RNase H⁻ reverse transcriptase (Invitrogen), and 5' end-labeled primer TO4 (5'-TGAACAGTGAGGCTCCCAT-3', 1 \times 10⁶ cpm) for 60 min at 50°C. The reaction mixture was formulated as recommended by the supplier. After the phenol-chloroform extraction, the products were separated on a 6% polyacrylamide/7 M urea gel, and the signals were detected by using a bioimage analyzer.

Results

AdoMet Down-Regulates *CGS1* Expression *in Vivo*. We have reported (8) that *CGS1* expression is feedback-regulated at the level of mRNA stability in response to methionine application, and that the exon 1 coding region of *CGS1* is necessary and sufficient for the posttranscriptional regulation *in vivo* (8, 10). In these experiments, however, the applied methionine may have affected *CGS1* expression after it was metabolized. Because AdoMet is the immediate metabolite of methionine (Fig. 1), we tested the effects of AdoMet on *CGS1* exon 1-mediated down-regulation by using a transient expression system.

The plasmids pMI4(WT) and pMI4(*mtol-1*) carry the WT and *mtol-1* mutant exon 1 coding sequence of *CGS1*, respectively, joined in-frame to the *Escherichia coli* β -glucuronidase (GUS) reporter gene, and placed under the control of a cauliflower mosaic virus 35S RNA promoter (8). The plasmid DNA was introduced into WT *A. thaliana* protoplasts by electroporation, and GUS reporter activity was analyzed relative to the LUC activity from a cotransfected control plasmid lacking the *CGS1* exon 1. For pMI4(WT), addition of 1 mM AdoMet resulted in reduced reporter activity (Fig. 2A). AdoMet application did not appreciably affect the reporter activity in tests containing pMI4(*mtol-1*) (Fig. 2A). These results suggested that, rather than methionine itself, AdoMet or one of its metabolites acts as the effector of *CGS1* regulation.

In another experiment designed to test the effect of AdoMet *in vivo*, we used protoplasts from the *mtol-1* and *mtol-3-1* mutants.

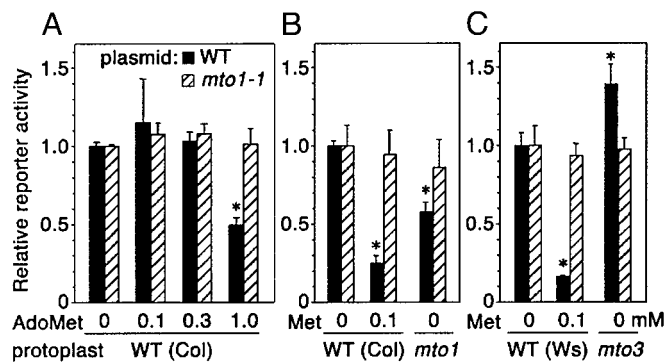


Fig. 2. Effects of AdoMet application and *mto* mutations in transient expression systems. Protoplasts were electroporated with pMI4(WT) (filled bars) or pMI4(*mto1-1*) (hatched bars) plasmid DNA, and were incubated for 48 h. GUS activity was normalized with LUC activity from the cotransfected 221-LUC+ plasmid, which does not carry the *CGS1* exon 1. Reporter activity relative to that of untreated WT protoplasts was calculated. Averages \pm SD of triplicate experiments are shown. *, values that were significantly different from those of untreated WT protoplasts ($P < 0.05$ by *t* test). (A) Effect of AdoMet treatment of Col-0 protoplasts. The electroporated protoplasts were treated with 0.1–1 mM AdoMet as indicated. (B) Effect of *mto1-1* mutation. DNAs were electroporated into protoplasts of the *mto1-1* mutant (*mto1*) and its parental WT Col-0. In the case of WT protoplasts, the effect of 0.1 mM methionine treatment was also analyzed. (C) Treatment was as in B, except that protoplasts from the *mto3-1* mutant (*mto3*) and its parental WT Ws were used.

The *mto1-1* mutant has a defect in the negative feedback regulation of *CGS1* (8), whereas the *mto3-1* mutant has a defect in an AdoMet synthetase (13). Both mutants overaccumulate methionine, but their accumulation of AdoMet differs. Compared with the WT, the *mto1-1* mutant has three times the level of endogenous AdoMet (15), whereas *mto3-1* has $\approx 70\%$ (13). The reporter activity from pMI4(WT) transfected into *mto1-1* mutant protoplasts was significantly lower than that in protoplasts from its WT strain Col-0 (Fig. 2B). In contrast, in *mto3-1* mutant protoplasts, the reporter activity was significantly higher than in protoplasts from its WT strain Ws (Fig. 2C). In both cases, reporter activity was not appreciably affected when pMI4(*mto1-1*) was used (Fig. 2B and C). The reporter activity of pMI4(WT) responded to methionine application in both Col-0 and Ws WT protoplasts, indicating that the inability of the *mto3-1* mutant to down-regulate the reporter activity is not due to the difference in ecotype. These data support the idea that, rather than methionine, AdoMet or one of its metabolites is the effector.

Establishment of the Wheat Germ *In Vitro* Translation System as the Experimental System. During *in vivo* tests of the effects of exogenously applied compounds, the efficiency of metabolite uptake into the cells can complicate interpretation of the results. We therefore exploited the *in vitro* translation system by using wheat germ extract to determine the actual effector of the regulation mediated by *CGS1* exon 1.

The exon 1 coding sequence of *CGS1* was joined in-frame to a LUC reporter gene, transcribed *in vitro* in the presence of the cap analog, and this *Ex1(WT):Luc* RNA, or its *mto1-1* mutant derivative *Ex1(mto1-1):Luc*, was translated in wheat germ extract. Capped *Rluc* RNA lacking *CGS1* exon 1 was simultaneously translated, and was used as an internal control. As shown in Fig. 3A, addition of up to 1 mM methionine to the *in vitro* translation system did not affect the reporter activity generated by *Ex1(WT):Luc* RNA, whereas addition of AdoMet at concentrations >0.1 mM down-regulated the reporter activity. Neither methionine nor AdoMet affected the reporter activity when

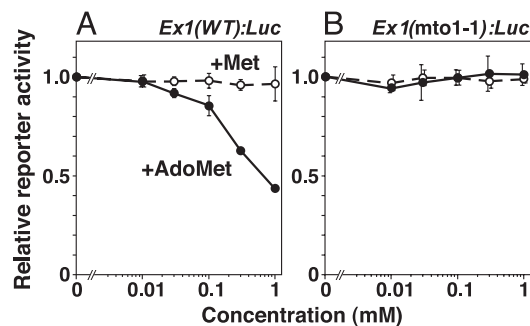


Fig. 3. Effects of methionine and AdoMet on reporter activity in the *in vitro* translation system. *Ex1(WT):Luc* RNA (A) or *Ex1(mto1-1):Luc* RNA (B) were translated in wheat germ extract for 120 min in the presence of various concentrations of AdoMet (●) or methionine (○) as indicated. LUC activity was normalized with the control RLuc activity of the cotranslated *Rluc* RNA, which does not carry the *CGS1* exon 1. Reporter activity relative to that in samples not treated with methionine or AdoMet was calculated, and averages \pm SD of triplicate experiments are shown.

Ex1(mto1-1):Luc RNA was used (Fig. 3B). These results suggest that AdoMet or one of its metabolites acts as the effector of this down-regulation *in vitro*.

The *mto1* alleles *mto1-2*, *mto1-3*, and *mto1-4* (9) were also tested (Table 1), revealing that the reporter activity is unchanged or only weakly responds to AdoMet, if *CGS1* exon 1 harbors any one of these mutations. The *mto1-4* mutation, which has been shown to be a leaky allele *in vivo* (9), also showed significantly lower reporter activity than other *mto1* alleles *in vitro*.

Identification of AdoMet as the Effector of *CGS1* Regulation. Because it is possible that some of the enzymes responsible for AdoMet metabolism are present in the wheat germ extract system, a number of compounds metabolically related to AdoMet were tested. When AdoMet is used in methyl transfer reactions, it is converted to AdoHcy, and AdoHcy is then recycled back to methionine through L-homocysteine (Fig. 1). Neither AdoHcy nor L-homocysteine affected the reporter activity (Fig. 4A), indicating that the metabolites of this recycling pathway are not involved in the regulation.

Both low-molecular-weight compounds and macromolecules act as acceptors of AdoMet-mediated methyl transfer reactions. It is possible that a product of these methyl transfer reactions acts as the effector. AdoHcy has been shown to be a potent competitive inhibitor of a number of methyltransferases that use AdoMet as the methyl donor (16). We therefore tested whether AdoHcy competes with AdoMet in the down-regulation of

Table 1. Response of *mto1* alleles to AdoMet in the *in vitro* translation system

RNA	Relative reporter activity (no added AdoMet = 1)
<i>Ex1(WT):Luc</i>	0.49 \pm 0.03 ^a
<i>Ex1(mto1-1):Luc</i>	1.02 \pm 0.03 ^c
<i>Ex1(mto1-2):Luc</i>	0.94 \pm 0.04 ^c
<i>Ex1(mto1-3):Luc</i>	0.96 \pm 0.03 ^c
<i>Ex1(mto1-4):Luc</i>	0.83 \pm 0.03 ^b

Ex1:Luc RNA carrying the WT or *mto1* mutant *CGS1* exon 1 was translated in wheat germ extract for 120 min in the presence of 1 mM AdoMet. LUC activity was normalized with the RLuc activity from the cotranslated *Rluc* RNA not carrying the *CGS1* exon 1 sequence, and reporter activity relative to samples with no added AdoMet was calculated. Averages \pm SD of triplicate experiments are shown. Superscript letters indicate that the values are significantly different from each other ($P < 0.05$ by *t* test).

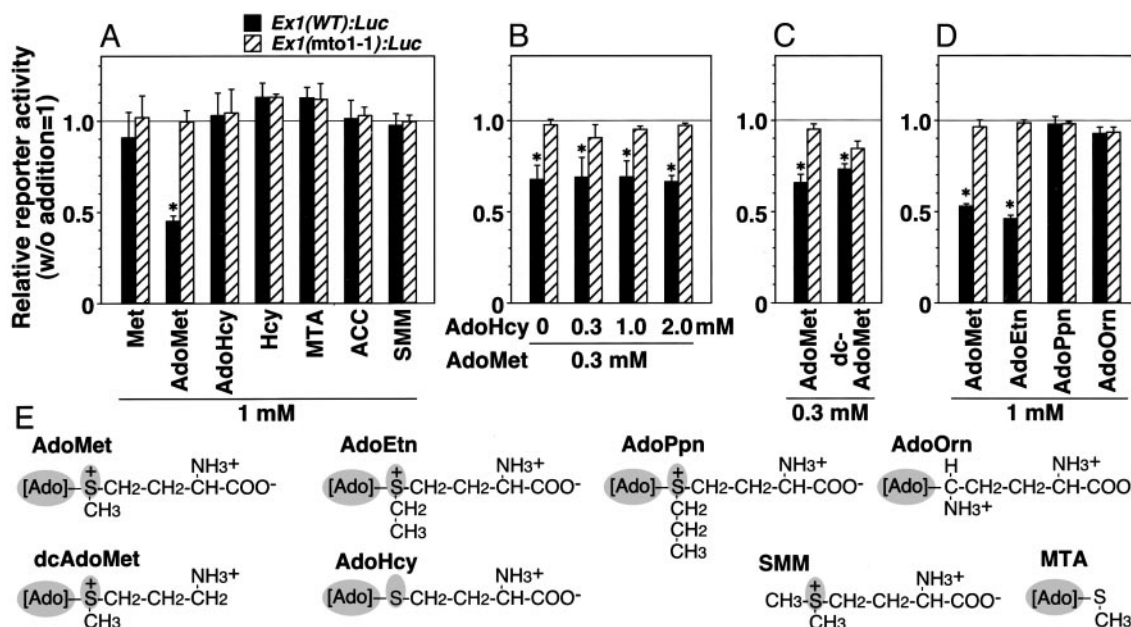


Fig. 4. Effects of metabolites and analogs of AdoMet on reporter activity *in vitro*. (A) Effects of AdoMet metabolites. *Ex1(WT):Luc* RNA (filled bars) or *Ex1(mto1-1):Luc* RNA (hatched bars) were translated in wheat germ extract for 120 min in the presence of 1 mM of various metabolites of AdoMet. LUC activity was normalized with the control RLuc activity from the cotranslated *Rluc* RNA, and reporter activity relative to samples without added metabolites was calculated. Averages \pm SD of triplicate experiments are shown. *, the value for *Ex1(WT):Luc* RNA is significantly different from that for *Ex1(mto1-1):Luc* RNA ($P < 0.05$ by *t* test). Abbreviations are the same as in Fig. 1. (B) Effect of AdoHcy on AdoMet-mediated down-regulation. *In vitro* translation was carried out in the presence of 0.3 mM AdoMet plus various concentrations of AdoHcy. (C) Effect of decarboxylated AdoMet (dcAdoMet). *In vitro* translation was carried out in the presence of 0.3 mM dcAdoMet. (D) Effects of analogs of AdoMet. *In vitro* translation was carried out in the presence of analogs at 1 mM. AdoEtn, S-adenosyl-L-ethionine; AdoPpn, S-adenosyl-L-propionine; and AdoOrn, adenosyl-L-ornithine. (E) Schematic representation of compounds used in this study that are metabolically and/or structurally related to AdoMet. The 5'-deoxyadenosine moiety (Ado) and the sulfonium sulfur (S^+) are shaded. Abbreviations are the same as in A–D.

reporter activity. AdoMet was added to 0.3 mM, and the concentration of AdoHcy was varied between 0.3 and 2 mM. As shown in Fig. 4B, AdoHcy exhibited no competitive effect on the AdoMet-mediated down-regulation of reporter activity, even when AdoHcy was added at ≈ 6 -fold the concentration of AdoMet. Because inhibitor constants for AdoHcy are usually about equal to, or lower than, the K_m values for AdoMet (16), it is unlikely that any of the methylated products, such as a methylated protein factor, acts as the effector of this regulation.

AdoMet is used in the biosynthesis of polyamines after it is decarboxylated by AdoMet decarboxylase. In this reaction, the methylthio moiety is recycled back to methionine through 5'-deoxy-5'-(methylthio)adenosine (Fig. 1). Because 1 mM dcAdoMet was inhibitory to the *in vitro* translation reaction (data not shown), the effect of this compound was tested at 0.3 mM. As shown in Fig. 4C, dcAdoMet down-regulated reporter activity, but the effect was $\approx 30\%$ that of AdoMet. Addition of spermidine and spermine did not appreciably affect relative reporter activities (data not shown). 5'-Deoxy-5'-(methylthio)adenosine also did not affect the reporter activity (Fig. 4A).

Other metabolites that diverge from AdoMet include 1-aminocyclopropane-1-carboxylic acid (17) and SMM (ref. 18 and Fig. 1). Neither compound appreciably affected reporter activity (Fig. 4A). These results support the idea that AdoMet, and not a metabolite of AdoMet, is the effector.

AdoEtn, an Analog of AdoMet, also Down-Regulates Reporter Activity.

To obtain insight into the structural features of the effector, the effects of AdoMet analogs were tested. AdoEtn has a sulfur-linked ethyl group in place of the methyl group of AdoMet (Fig. 4E). As shown in Fig. 4D, AdoEtn down-regulated reporter activity to a similar extent as that of AdoMet. We also tested

AdoPpn and AdoOrn, which have a sulfur-linked propyl group and a carbon-linked amino group, respectively, in place of the sulfur-linked methyl group of AdoMet (Fig. 4E). Neither compound affected reporter activity (Fig. 4D).

Identification of a 5'-Truncated RNA Species After *In Vitro* Translation.

We have reported (8) that when methionine is applied to WT *A. thaliana* calli, accumulation of *CGSI* mRNA is reduced, and a related short RNA species that is truncated at its 5' end appears. We have suggested that this 5'-truncated RNA is a degradation intermediate of *CGSI* mRNA. A similar RNA species was also detected in methionine-treated transgenic *A. thaliana* carrying essentially the same construct as pMI4(WT) (10), which indicates that the exon 1 coding sequence of *CGSI* alone is sufficient to produce the 5'-truncated RNA.

We tested whether the 5'-truncated RNA is produced in our *in vitro* system. RNA was analyzed after translation in wheat germ extract in the presence or absence of 1 mM AdoMet. Northern hybridization was carried out after poly(A) selection. As shown in Fig. 5A, the *luc+* probe detected an RNA species ≈ 300 bases shorter than the full-length RNA when *Ex1(WT):Luc* RNA was translated in the presence of AdoMet. This short RNA band was also detected by the probe covering the 3' region of the *luc+* reporter gene (Fig. 5B), but not with the probe covering the 5' region of the *CGSI* exon 1 coding sequence (Fig. 5C), indicating that it is truncated at its 5' end. The amounts of the full-length RNA remaining after the *in vitro* reaction were not significantly different between the presence and absence of AdoMet. *Ex1(mto1-1):Luc* RNA (Fig. 5A) and cotranslated *Rluc* RNA (Fig. 5D) did not produce a truncated RNA species. Northern hybridization of total RNA produced

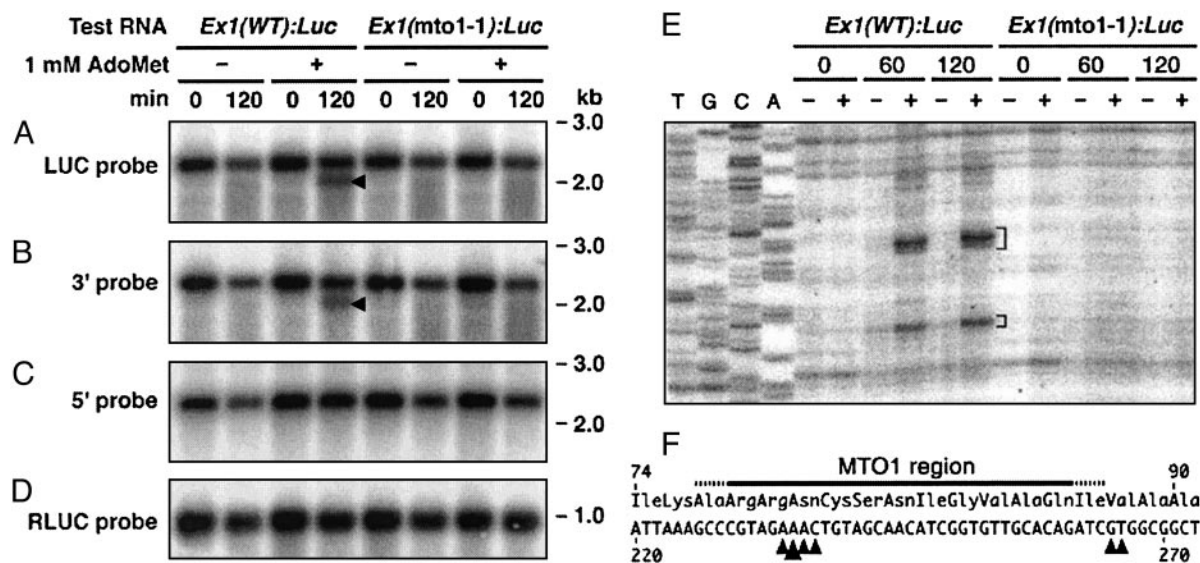


Fig. 5. Analysis of RNA after *in vitro* translation. (A–D) *Ex1(WT):Luc* RNA or *Ex1(mto1-1):Luc* RNA were translated in wheat germ extract in the presence (+) or absence (–) of 1 mM AdoMet. Poly(A) RNA was extracted immediately after (0 min) or 120 min after the start of *in vitro* translation, and was fractionated in 1% agarose gels containing formaldehyde. Northern hybridizations were carried out by using a ³²P-labeled *luc+* gene probe (A), a probe covering the 3' region of the *luc+* coding sequence (B), or a probe covering the 5' region of the *CGS1* exon 1 coding sequence (C). Northern hybridization was also carried out by using an RLUC probe to detect the cotranslated *Rluc* RNA (D). Positions of size markers are indicated to the right. Arrowheads in A and B indicate the positions of the 5'-truncated RNA. Representative results of triplicate experiments are shown. (E) Detection of the 5' ends of truncated RNA species by primer extension. Poly(A) RNA was prepared as in A, and primer extension reactions were carried out by using TO4 primer. Lanes T, G, C, and A are the sequence ladder resulting from the same primer. Brackets indicate the 5' ends of the truncated RNA. (F) The 5' ends were mapped on the nucleotide (numbered from the first ATG) and the amino acid sequence. Arrowheads indicate positions of the 5' ends with the large arrowhead pointing to the most prominent 5' end. The MTO1 region (9) is marked above the amino acid sequence. Similar results were obtained in quadruplicate experiments.

similar results to those of poly(A)-selected RNA (data not shown).

To map the 5' end of the truncated RNA, we carried out primer extension studies. When *Ex1(WT):Luc* RNA was translated in the presence of AdoMet, the 5' end of the truncated RNA resolved into two regions 234–237 and 265–266 bases from the first ATG, with position 235 most prominent (Fig. 5 E and F). These 5' ends were located within or very close to the MTO1 region (9). Primer extension studies using poly(A) RNA from methionine-treated WT calli showed that at least the most prominent 5' end of the truncated RNA *in vitro* was also detected *in vivo*, suggesting that the truncated RNA produced in these two systems are essentially the same (see Supporting Text and Fig. 6, which are published as supporting information on the PNAS web site, www.pnas.org.).

Discussion

Identification of AdoMet as the Natural Effector of the Posttranscriptional Regulation Mediated by *CGS1* Exon 1. Transient expression experiments suggested that, rather than methionine, AdoMet or one of its metabolites is the effector of *CGS1* regulation *in vivo*. The results corroborate previous findings (13, 19) with the *mto3* mutants and cosuppression lines of the AdoMet synthetase gene in *A. thaliana* (20). In these systems, the steady-state level of *CGS1* mRNA was the same as, or greater than, that in WT, despite the fact that these plants overaccumulate methionine. In AdoMet feeding experiments, 1 mM AdoMet was less effective in down-regulating expression than was 0.1 mM methionine (compare Fig. 2 A and B). This may be due, however, to AdoMet being taken up less efficiently into cells or being more labile in the experimental medium than in methionine. Although AdoMet transporters have been characterized from *Saccharomyces cerevisiae* (21), homologous transporters do not appear to exist in the *A. thaliana* genome.

In this article, a wheat germ *in vitro* translation system was

used to identify the effector of the regulation mediated by *CGS1* exon 1. A number of different metabolites related to AdoMet were tested, and AdoMet was found to be the most effective in blocking the reporter activity. dcAdoMet was also effective, although weakly. However, because the concentration of dcAdoMet *in vivo* has been reported to be ≈2% of that of AdoMet (22), it is unlikely that this metabolite exerts a greater effect than AdoMet *in vivo*. Our results establish that AdoMet is the natural effector of the posttranscriptional regulation mediated by the exon 1 of *CGS1*, and suggest that in the *in vivo* experiments (8–10), the down-regulation was induced after methionine was converted to AdoMet by AdoMet synthetase.

AdoEtn, an analog of AdoMet, was shown to be as effective as AdoMet in down-regulating reporter activity. AdoMet has an α-aminobutyric acid moiety, a 5'-deoxyadenosine moiety, and a methyl group linked to the sulfonium sulfur (Fig. 4E). AdoEtn differs from AdoMet by containing a sulfur-linked ethyl group (Fig. 4E). Therefore, the regulation mechanism tolerates either a methyl or an ethyl group. However, a propyl group is not tolerated at this position, because AdoPpn (Fig. 4E) was not effective. AdoHcy lacks the sulfur-linked alkyl group (Fig. 4E) and was also ineffective, suggesting that the sulfonium group is a necessary component of effector. The fact that AdoOrn (Fig. 4E) had no effect supports this notion. Although SMM is a sulfonium compound (Fig. 4E), it had no effect on reporter activity, demonstrating the importance of the 5'-deoxyadenosine moiety. The carboxylic acid group is necessary, but is less important than the sulfonium and 5'-deoxyadenosine moieties, as dcAdoMet was a weak effector.

It was recently reported (23) that in a wheat germ *in vitro*-coupled transcription/translation system, methionine down-regulated the accumulation of transcripts from the full-length *CGS1* cDNA, although production of the 5'-truncated RNA species was not discussed. Regions of *CGS1* other than exon 1 might carry additional regulatory functions, however, the re-

ported response should represent a minor component *in vivo* as it does not corroborate the *CGSI* mRNA levels in the methionine overaccumulation lines discussed above (13, 19, 20). In our *in vitro* system, addition of 1 mM methionine had no effect on the RNA profile (data not shown).

Use of the Wheat Germ *in Vitro* Translation System to Study the Mechanism of *CGSI* Regulation. Two lines of evidence suggest that the reactions that we observed in the wheat germ *in vitro* translation system reflect the posttranscriptional regulation of *CGSI* *in vivo*. First, the response to AdoMet was strongly reduced when the *CGSI* exon 1 carried one of the *mtol* mutant alleles. In addition, *Ex1(mtol-4):Luc* RNA showed significantly lower reporter activity than those RNAs that carry the other *mtol* alleles (Table 1). The *mtol-4* mutant has been shown to be a leaky allele *in vivo* (9). This finding means that the phenotypic differences identified genetically *in vivo*, and the level of response *in vitro*, are interrelated. Second, analysis of *Ex1(WT):Luc* RNA after *in vitro* translation in the presence of AdoMet, revealed the presence of a 5'-truncated RNA species, which we have suggested to be a degradation intermediate (8, 10). In addition, the 5' end of the truncated RNA species produced *in vivo* and *in vitro* were essentially the same. These data suggest that the same RNA degradation event that occurs *in vivo* also occurs *in vitro*.

In contrast, the amounts of full-length *Ex1(WT):Luc* RNA that remained after *in vitro* translation in the presence and absence of AdoMet did not match the difference in reporter activities. This result suggests that the down-regulation of reporter activity is not a direct consequence of mRNA degradation. It is possible that the down-regulation of reporter activity that we observed *in vitro* could have mainly reflected a translational repression. In some eukaryotic genes, translational regulation by a peptide encoded by an upstream ORF is known (24). Although the regulation is not linked to mRNA degradation, the mechanism shares similarity to the *CGSI* system in that a short stretch of amino acid sequence acts in *cis*. Unlike the *CGSI* system, the regulation by an upstream ORF is usually exerted at

the termination of translation. However, in the case of the arginine attenuator peptide in *arg-2* of *Neurospora crassa* and its *S. cerevisiae* counterpart, which encode an enzyme for arginine biosynthesis, the stop codon is dispensable, and *in vitro* translation systems reproduced the response (25, 26). Among the genes whose expression is regulated at the level of mRNA stability, there are cases where the stability determinants are located in their coding sequences (27–31). One such gene is *c-myc*, for which *in vitro* studies have been applied. Studies using rabbit reticulocyte lysate from which tRNA was depleted by nuclease treatment have shown that *c-myc* translation pauses at one of the rare codons, and suggested that this triggers *c-myc* mRNA degradation (28). However, it is unlikely that rare codons are involved in the regulation of *CGSI*, because among the 40 *in vivo*- and *in vitro*-generated mutants that we have obtained with lesions within the *CGSI* exon 1 (8, 9), there was no correlation between codon usage and phenotype (data not shown), and the *in vitro* translation system used in this article contained ample amounts of added tRNA.

The data presented in this article suggest that the basic reactions of *CGSI* regulation occur in the wheat germ *in vitro* translation system. This system allows us to examine the molecular mechanisms of posttranscriptional regulation of *CGSI*, by providing a biochemical strategy to complement the genetic advantages of the *A. thaliana* system, which has proven amenable in study of the control of mRNA stability (8, 32).

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