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A halt in poly(A) shortening during *S*-adenosyl-L-methionine-induced translation arrest in *CGS1* mRNA of *Arabidopsis thaliana*

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Cystathionine γ-synthase (CGS) catalyzes the first committed step of methionine (Met) biosynthesis in plants. Expression of the *Arabidopsis thaliana CGS1* gene is negatively feedback-regulated in response to the direct Met metabolite *S*-adenosyl-L-methionine (AdoMet). This regulation occurs at the step of mRNA stability during translation and is coupled with AdoMet-induced *CGS1*-specific translation arrest. In general, mRNA decay is initiated by a shortening of the poly(A) tail. However, this process has not been studied in detail in cases where regulatory events, such as programmed translation arrest, are involved. Here, we report that the poly(A) tail of the full-length *CGS1* mRNA showed an apparent increase from 50–80 nucleotides (nt) to 140–150 nt after the induction of *CGS1* mRNA degradation. This finding was unexpected because mRNAs that are destined for degradation harbored longer poly(A) tail than mRNAs that were not targeted for degradation. The results suggest that poly(A) shortening is inhibited or delayed during AdoMet-induced translation arrest of *CGS1* mRNA. We propose an explanation for this phenomenon that remains consistent with the recent model of actively translating mRNA. We also found that *CGS1* mRNA degradation intermediates, which are 5'-truncated forms of *CGS1* mRNA, had a short poly(A) tail of 10–30 nt. This suggests that poly(A) shortening occurs rapidly on the degradation intermediates. The present study highlights *CGS1* mRNA degradation as a useful system to understand the dynamic features of poly(A) shortening.

Key words: *Arabidopsis thaliana*, methionine biosynthesis, mRNA degradation, poly(A) shortening, RNase H digestion

INTRODUCTION

Studies on the control of gene expression have focused on transcriptional regulation; however posttranscriptional regulation is also important for modulating gene expression (Moore, 2005; Wiederhold and Passmore, 2010). In particular, control of mRNA stability has recently been recognized as an important regulatory process in a number of genetic systems, especially in response to changes in external and intra-cellular conditions. A growing number of reports have shown the involvement of mRNA stability control in plants in response to environmental changes such as temperature, nutrient conditions, osmotic stress, and pathogen infection (Molin et al., 2009; Romero-Santacreu et al., 2009; Castells-Roca et al., 2011; Miller et al., 2011; Rabani et al., 2011; Tanaka et al., 2011; Chiba et al., 2013).

Cystathionine γ-synthase (CGS; EC 2.5.1.48) catalyzes the first committed step of methionine (Met) biosynthesis in plants (Matthews, 1999), and is encoded by the *CGS1* gene in *Arabidopsis thaliana* (Kim and Leustek, 1996; gene ID At3g01120). Unlike many of the regulatory step enzymes in metabolic pathways, CGS is not an allosteric enzyme (Thompson et al., 1982) and its activity is negatively feedback-regulated at the level of mRNA stability in response to *S*-adenosyl-L-methionine (AdoMet), a direct metabolite of Met. Upon the induction of *CGS1* mRNA degradation by AdoMet, 5'-truncated RNA species of *CGS1* are formed as degradation intermediates (Chiba

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et al., 1999, 2003; Haraguchi et al., 2008).

A short amino acid sequence, termed the MTO1 region, 77-RRNCSNIGVAQ-87, encoded in the N-terminal region of *CGS1*, acts as the *cis*-element for this regulation (Ominato et al., 2002; Chiba et al., 2003). *A. thaliana mto1* mutants that bear single amino acid sequence alterations within the MTO1 region abolish this feedback regulation, resulting in an over-accumulation of soluble Met (Ominato et al., 2002; for review, see Goto et al., 2005).

Translation is required for the AdoMet-induced degradation of *CGS1* mRNA (Lambein et al., 2003), which can be recapitulated in the wheat germ extract (WGE) *in vitro* translation system (Chiba et al., 2003). Studies using WGE revealed that, prior to mRNA degradation, AdoMet induces a temporal arrest of translation elongation at the Ser-94 codon located immediately downstream of the *MTO1* region, and that an mRNA degradation event occurs near the 5'-edge of the arrested ribosome (Onouchi et al., 2005). Primer extension studies detected several 5'-ends for the degradation intermediates, which are separated by ~30 nucleotides (nt) from each other (Haraguchi et al., 2008). The two shortest intermediates are also detectable *in vivo* (Chiba et al., 2003; Fig. 1A). *In vitro* experiments showed that upon translation arrest, ribosomes are stacked behind the initially arrested ribosome, and suggested that the 5'-ends of the degradation intermediates correspond to these stalled ribosomes (Haraguchi et al., 2008). However, the molecular mechanism of AdoMet-induced *CGS1* mRNA degradation remains to be elucidated.

mRNA degradation in eukaryotes has been studied extensively in yeast both genetically and biochemically (for review, see Parker, 2012). It is generally accepted that higher eukaryotes, including plants, share the basic mechanisms of mRNA degradation with yeast. According to the current understanding in higher eukaryotes, mRNA is predominantly degraded by two alternative pathways; a deadenylation-dependent decapping pathway and a 3' to 5' decay pathway. Poly(A) shortening is the first, and usually the rate-limiting, step in both pathways. Deadenylated mRNA is either degraded by 5'–3' exoribonuclease following decapping in the former pathway, or is degraded in a 3' to 5' direction in the latter pathway (for reviews, see Parker and Song, 2004; Chiba and Green, 2009; Houseley and Tollervey, 2009). Poly(A) length plays a critical role in determining the fate of mRNAs due to its effects on basal mRNA turnover as well as on the specific regulation of mRNA degradation and translation (Wiederhold and Passmore, 2010; Weill et al., 2012). Computational simulation of the mRNA degradation process has also suggested that deadenylation is the most effective step for the control of mRNA stability (Cao and Parker, 2001). Therefore, characterizations of changes in the poly(A) length are crucial to understanding the control of mRNA stability.

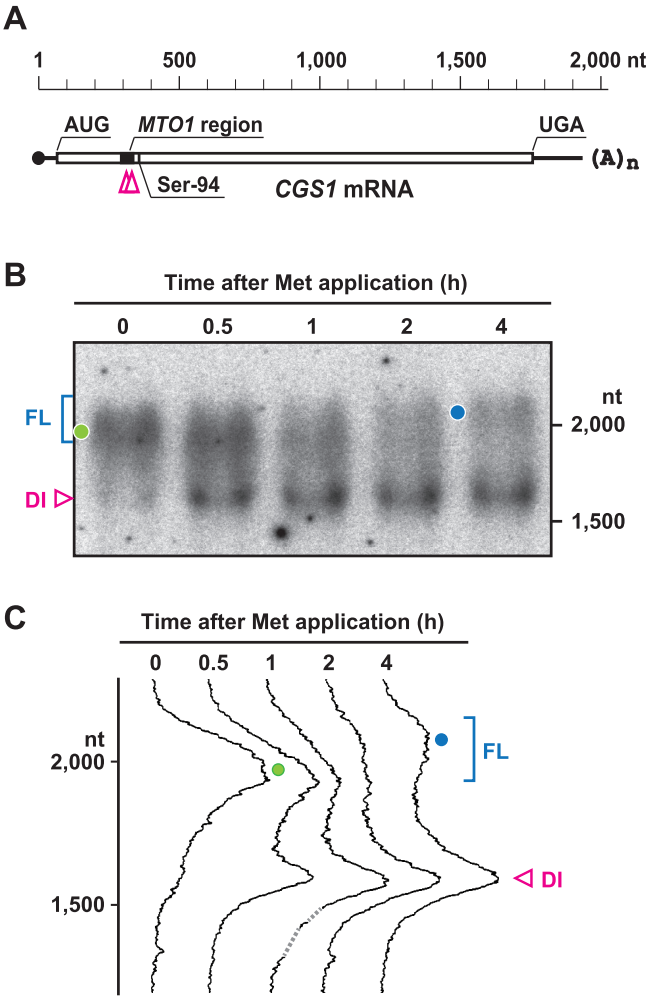

Fig. 1. Changes in the length of *CGS1* mRNA after Met application. (A) Schematic representation of *CGS1* mRNA. The body of the full-length *CGS1* mRNA is 1,935 nt and includes a 65 nt 5'-untranslated region (UTR) (the RIKEN *Arabidopsis* full-length cDNA clone, RAFL09-59-O05; Sakurai et al., 2005) and 178 nt 3'-UTR (Kim et al., 1999). The coding region of 563 amino acids from AUG to UGA (Kim and Leustek, 1996) is shown as an open box. AdoMet-induced translation arrest occurs at the Ser-94 codon located immediately downstream of the *MTO1* region (filled box). Open arrowheads indicate the 5'-ends of the degradation intermediates that we previously reported in wild-type calli after Met application, which differ in size by ~30 nt (Chiba et al., 2003). (B) Total RNA was extracted from wild-type calli at the indicated time points after application of 1 mM Met. Samples were size-fractionated on a 1.2% (w/v) agarose-formaldehyde gel and subjected to Northern hybridization using a ^{32}P-labeled *CGS*ΔEx1 probe. The peak positions of the full-length mRNA (FL) before (green) and 4 h after (blue) Met application are marked with circles. The position of degradation intermediates (DI) is marked with an open arrowhead. Similar results were obtained in duplicate experiments. (C) Scanned image of (B) is presented. Positions of mRNA peaks are marked as in (B). Obvious noise peaks due to dirt spots were eliminated (dashed lines).

During the course of *in vivo* studies on *CGS1* mRNA degradation, we noticed an unusual shift in the size of full-length *CGS1* mRNA when *CGS1* mRNA degradation

was induced. In the present study, we specifically focused on changes in poly(A) length, and we found that *CGS1* mRNA harbored a longer poly(A) tail after the induction of *CGS1* mRNA degradation than before the induction. The finding suggests that *CGS1* mRNA is protected from poly(A) shortening during AdoMet-induced translation arrest. The mechanism of this phenomenon is discussed in the light of the recent understanding of actively translating mRNA.

MATERIALS AND METHODS

***A. thaliana* callus cultures and Met application** Liquid callus cultures of wild-type or *mto1-1* mutant *A. thaliana* (ecotype Col-0) were prepared as described (Chiba et al., 1999). Treatment with 1 mM Met (Wako Pure Chemicals) was carried out as described previously (Lambein et al., 2003). Samples were briefly dried on filter paper, frozen in liquid nitrogen and stored at –80°C until RNA extraction.

Total RNA extraction and Northern hybridization Total RNA was extracted from the calli using an RNeasy Plant Mini kit (Qiagen) after homogenization of samples in a Mixer Mill MM300 (Qiagen). Total RNA (10 μg) was separated on agarose-formaldehyde gels prior to transfer to nylon membranes (Gene Screen Plus, NEN Life Science Products). Northern hybridization was carried out as described previously (Chiba et al., 1999). The *CGS* ΔEx1 probe has been described (Suzuki et al., 2001). Poly(A) selection was not applied to the RNA preparations used in this study, so that RNA species with short poly(A) tails would be retained.

Determination of poly(A) tail length by RNase H digestion Total RNA (30 μg) was mixed with 125 ng oligodeoxyribonucleotide(s) RnH1500 (5'-CTGACCACACCTCCAAAACCAG-3') and/or oligo(dT)$_{12–18}$ (Invitrogen) and denatured at 95°C for 5 min, followed by rapid cooling on ice for 5 min. For detection of the poly(A) tail without trimming the 5' region, the RnH1500 oligodeoxyribonucleotide was omitted. In a reaction volume of 75 μl, 7.5 μl 10× RnH buffer (500 mM Tris·HCl pH 8.0, 100 mM $MgCl_2$, 1 M NaCl) was added and the samples were incubated at 42°C for 15 min to anneal the oligodeoxyribonucleotide(s) to RNA. After cooling on ice, 12.5 U of *E. coli* RNase H (Takara) was added and incubated at 37°C for 30 min. RNA samples (10 μg) were separated on agarose-formaldehyde gels and transferred to nylon membranes (Gene Screen Plus, NEN Life Science Products). Northern hybridization was carried out as described (Chiba et al., 1999).

Hybridization probes used were 3'-UTR, 3'-RnH and UBQ5. The 3'-UTR probe has been described (Chiba et al., 1999). To generate the 3'-RnH probe, total RNA extracted from three-week-old calli was amplified by a reverse transcription (RT)-PCR with the primers 5'-CTGGTTTTGGAGGTGTGGT-3' and 5'-GAACTAAACAGAATTTATTATATAGCACAAC-3'. The UBQ5 probe was generated by PCR amplification of genomic DNA with the primers 5'-GTGGTGCTAAGAAGAGGAAGA-3' and 5'-TCAAGCTTCAACTCCTTCTTT-3'. RT-PCR was carried out using a OneStep RT-PCR kit (Qiagen). Probes were labeled with [α-^{32}P]ATP (Hungarian Academy of Sciences) using the Megaprime DNA Labeling Systems (GE Healthcare).

Northern data acquisition and poly(A) length estimation Hybridization signals were detected with a BAS1000 BioImage Analyzer (Fuji Photo Film) or a Fluoro Imager FLA-7000 (Fuji Photo Film). Digital scanning data of the hybridization signals were obtained using the NIH Image and MultiGauge softwares. The data were normalized to the highest value in each lane to facilitate comparison of peak positions. The lengths of the RNAs were estimated by fitting the size marker positions to a linear line in a semi-log plot by the least-square method, and the poly(A) lengths were estimated by subtracting the mRNA length without poly(A) from that with poly(A). To detect the peak position, a moving average method was applied to smooth the data, when necessary. The "gel smiling" effect was corrected after comparing the peak positions at the right and left ends of each lane. The hybridization image contrast was adjusted using Photoshop software to enhance visibility.

RESULTS

***CGS1* mRNA of increased length accumulates when AdoMet-dependent *CGS1* mRNA degradation is induced** Although AdoMet is the direct effector of the feedback regulation of *CGS1* expression, Met application was used in this study to induce *CGS1* mRNA degradation because it is metabolized to AdoMet within the cell and is more efficient than AdoMet for *in vivo* experiments (Chiba et al., 2003). Met was applied to the liquid callus cultures of *A. thaliana*, and time-course samples of total RNA were subjected to Northern blot analysis to detect *CGS1* mRNA. Effects of Met application was followed for 4 h, because we have previously determined that repression of the full-length *CGS1* mRNA accumulation, as well as appearance of *CGS1* mRNA degradation intermediates, are evident by 2 h after Met application (Chiba et al., 1999; Lambein et al., 2003). Accumulation of full-length *CGS1* mRNA decreased gradually after Met application. In parallel with this decrease, the full-length *CGS1* mRNA showed an increase in length 2–4 h after Met application (Fig. 1, B and C).

Accumulation of *CGS1* mRNA degradation intermediates was evident as early as 0.5 h after Met application,

and the mobility of the degradation intermediates did not show any appreciable change during the 4-h experimental period. A faint degradation intermediate signal was also detected at 0 h (immediately prior to Met application), implying that a small fraction of *CGS1* mRNA is subject to the feedback regulation even under normal conditions. We have previously reported that, in Met-treated calli, two 5' ends are detected for the degradation intermediates by primer extension experiments, and are thought to be formed at the 5'-edge of the arrested and stacked ribosomes (Chiba et al., 2003; Onouchi et al., 2005; Haraguchi et al., 2008; Fig. 1A). These two degradation intermediates are detected as one band in Northern blots that are designed to detect full-length *CGS1* mRNA (~2,000 nt), as they differ in size by only ~30 nt.

The change in the length of full-length *CGS1* mRNA after Met application was unexpected. A number of possible mechanisms could lead to an increase in mRNA length including changes in the transcription start site and/or poly(A) addition site, alternative splicing, or changes in poly(A) length. In our previous primer extension experiments with *CGS1* mRNA prepared from Met-treated calli, we observed no detectable shift in the position of the full-length mRNA signal (Chiba et al., 2003), which eliminates the possibility of a change in the transcription start site.

Full-length *CGS1* mRNA harbors a longer poly(A) tail after Met application To evaluate the possible contribution of changes in the poly(A) tail length to the increase in full-length *CGS1* mRNA size, poly(A) tail lengths were compared before and after Met application. The poly(A) tail was removed by annealing with oligo(dT) followed by RNase H digestion. RNase H makes an endoribonucleolytic cleavage in the RNA moiety of a DNA:RNA duplex and then digests the RNA part exoribonucleolytically in the 3' to 5' direction (Schatz et al., 1990; Rizzo et al., 2002). The poly(A) lengths can then be compared by running samples with and without oligo(dT) on a gel for Northern hybridization (Fig. 2A).

As shown in Fig. 2B, total RNA from wild-type calli before (0 h) and 4 h after Met application showed that the size of the full-length *CGS1* mRNA was visibly reduced by the removal of poly(A) tail, suggesting that the full-length *CGS1* mRNA harbored a relatively long poly(A) tail. On the other hand, the poly(A) tail removal showed a minimal effect on the mobility of the degradation intermediate band, suggesting that the degradation intermediates harbor short poly(A) tails (Fig. 2B, lanes 3 and 4).

The data also showed that the size of the body of full-length *CGS1* mRNA after removal of the poly(A) tail was not appreciably different between the samples before and after Met application (Fig. 2B, lanes 2 and 4). This result is consistent with a negligible contribution from changes in the transcription start site, poly(A) addition

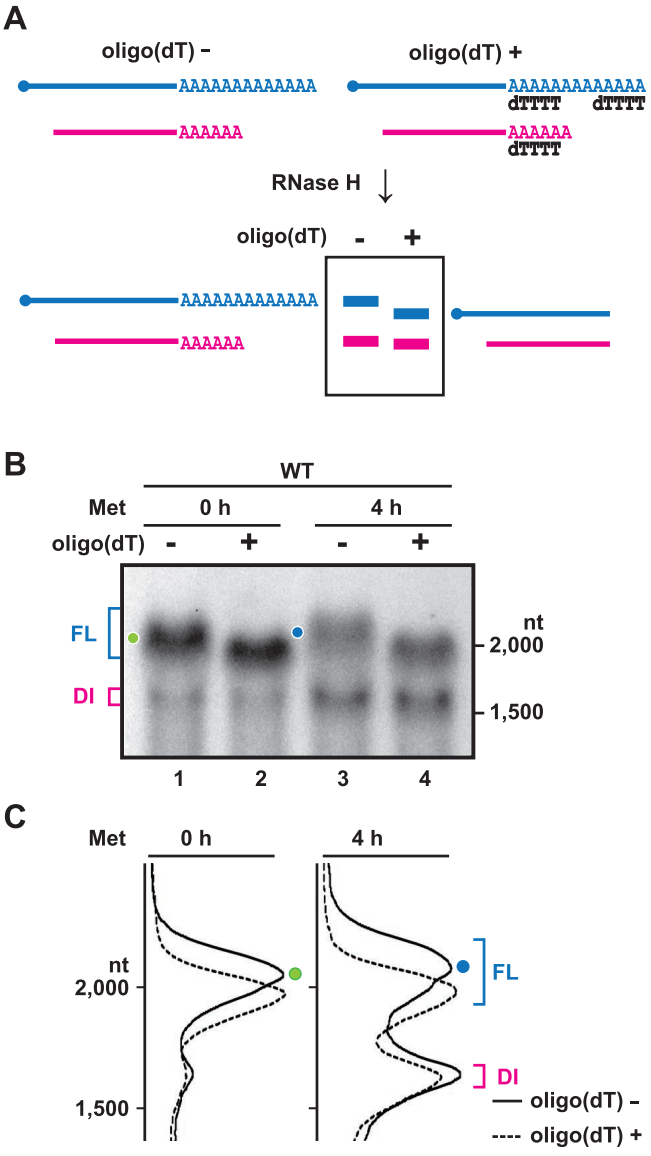

Fig. 2. Comparison of the poly(A) length of full-length *CGS1* mRNA by RNase H digestion. (A) Schematic presentation of a comparison of poly(A) lengths. Total RNA was annealed with oligo(dT) and subjected to RNase H digestion to remove the poly(A) tail. Poly(A) length can be determined by comparing the RNA size with or without the poly(A) tail. In this cartoon, differing poly(A) tail lengths are shown for the full-length mRNA and degradation intermediates to corroborate the experimental data in (B). (B) Total RNA was extracted from wild-type calli before (lanes 1, 2) and 4 h after 1 mM Met application (lanes 3, 4). Each sample was divided into two portions with (lanes 2, 4) or without (lanes 1, 3) oligo(dT), followed by RNase H digestion. The samples were size-fractionated on a 1.2% (w/v) agarose-formaldehyde gel and subjected to Northern hybridization analysis using a ^{32}P-labeled *CGS*ΔEx1 probe. Brackets mark the full-length (FL) and degradation intermediates (DI) of *CGS1* mRNA. Filled circles mark the peak positions of the full-length mRNA without eliminating the poly(A) tail before (green) and 4 h after (blue) Met application. Similar results were obtained in triplicate experiments. (C) Scanned image of (B) is presented after grouping the data with (dashed line) or without (solid line) oligo(dT) for each time point. Marks are the same as in (B).

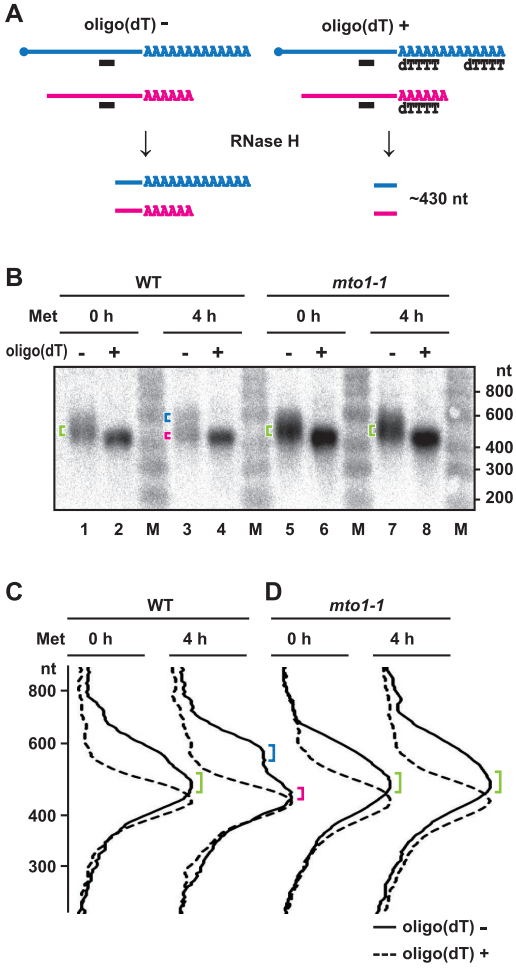

Fig. 3. Poly(A) tail length of *CGS1* mRNA before and after Met application in wild-type and *mto1-1* mutant calli. (A) Schematic presentation of poly(A) length determination after trimming the 5' region of *CGS1* mRNA by RNase H digestion. To make the poly(A) length difference readily detectable, total RNA was annealed with the RnH1500 deoxyoligonucleotide (black bar) that anneals to the 3' region of *CGS1* mRNA at nucleotide positions 1,486–1,507, or 449–428 nt upstream of the poly(A) addition site. A portion of the sample was also annealed with oligo(dT) as in Fig. 2A. RNase H digestion followed by Northern hybridization using a probe that hybridizes to the 3'-region will detect a band with a ~430 nt 3'-region plus the poly(A) tail in the "oligo(dT) –" sample, while the ~430-nt 3'-region alone will be detected in the "oligo(dT) +" sample. (B) Poly(A) lengths of *CGS1* mRNA in wild-type (lanes 1–4) and *mto1-1* mutant (lanes 5–8) calli were determined by RNase H digestion. Total RNA was extracted from the wild-type and *mto1-1* mutant calli before (lanes 1, 2, 5, 6) and 4 h after (lanes 3, 4, 7, 8) application of 1 mM Met. Each sample was mixed with the RnH1500 oligodeoxyribonucleotide, divided into two portions with (lanes 2, 4, 6, 8) or without (lanes 1, 3, 5, 7) oligo(dT), followed by RNase H digestion. The samples were size-fractionated on a 3% (w/v) agarose-formaldehyde gel and the *CGS1* mRNA 3'-region was detected by Northern hybridization using a ^{32}P-labeled 3'-UTR probe. Peaks of the bands in the samples from wild-type calli without oligo(dT) before (green) and after (magenta or blue) Met application are marked by brackets. The peaks of the *mto1-1* mutant *CGS1* mRNA are marked by green brackets. Similar results were obtained in triplicate experiments. Lanes M are size markers. (C, D) Scanned image of (A) is presented after grouping the data with (dashed line) and without (solid line) oligo(dT) for wild-type (C) and *mto1-1* mutant (D). Brackets mark the peaks as in (A).

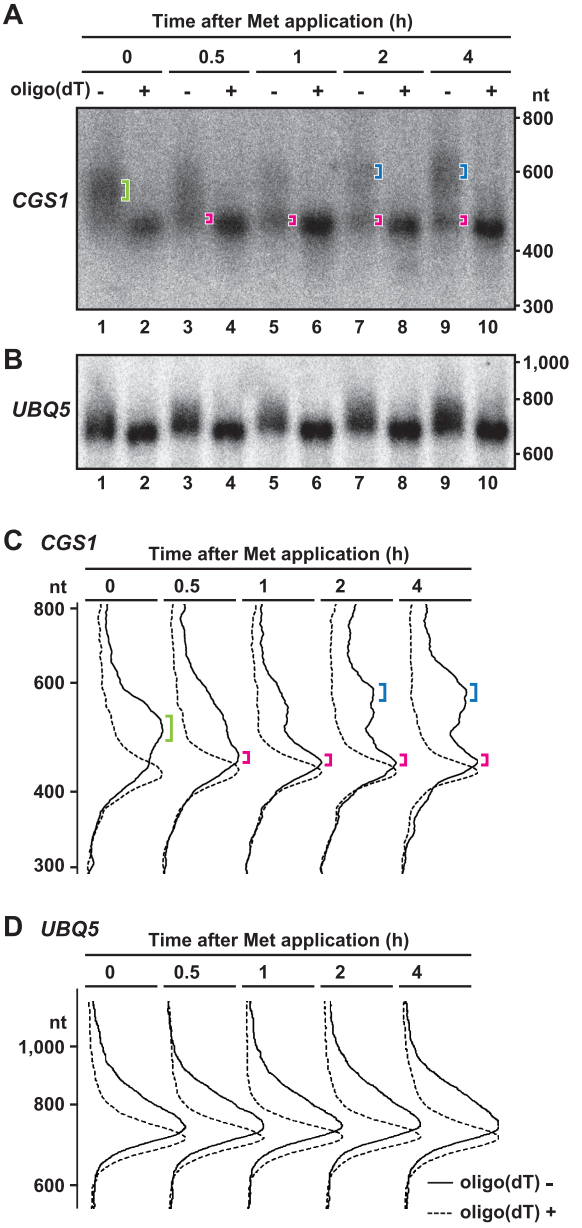

Fig. 4. Temporal changes in poly(A) tail lengths after Met application in *CGS1* and *UBQ5* mRNAs. (A) Poly(A) lengths of *CGS1* mRNA were determined by RNase H digestion (Fig. 3A). Total RNA was extracted from the wild-type calli at the indicated time points after application of 1 mM Met. Each sample was mixed with the RnH1500 oligodeoxyribonucleotide and divided into two portions with (lanes 2, 4, 6, 8, 10) or without (lanes 1, 3, 5, 7, 9) oligo(dT), followed by RNase H digestion. The samples were size-fractionated on a 3% (w/v) agarose-formaldehyde gel and the 3'-region of *CGS1* mRNA was detected by Northern hybridization using a ^{32}P-labeled 3'-RnH probe. Brackets mark the peaks of the bands in those samples without oligo(dT) before (green) and after (magenta and blue) Met application. (B) Poly(A) length of *UBQ5* mRNA was determined by rehybridizing the membrane used in (A) with a ^{32}P-labeled *UBQ5* probe. Trimming of the 5'-region was omitted because the full-length *UBQ5* mRNA is ~700 nt, which is sufficiently short for poly(A) length measurements. (C and D) Scanned images of (A) and (B), respectively, are presented after grouping the data with (dashed line) and without (solid line) oligo(dT) for each time point. In (C), the brackets mark the peaks as in (A).

site and alternative splicing.

Poly(A) length determination of the full-length and degradation intermediates of *CGS1* mRNA To evaluate the poly(A) lengths more precisely, the body of the *CGS1* mRNA was shortened by RNase H digestion with a *CGS1* mRNA-specific deoxyoligonucleotide that anneals ~430 nt upstream of the poly(A) addition site (Fig. 3A). The resulting bands were then detected by Northern hybridization using a probe that hybridizes to the 3' region of the *CGS1* mRNA.

Total RNA prepared from wild-type calli before (0 h) and 4 h after Met application were subjected to poly(A) length determination. For the samples from which the poly(A) tail was removed, Northern hybridization produced a band with a single peak of ~430 nt (Fig. 3B, lanes 2 and 4; Fig. 3C). On the other hand, for the samples from which the poly(A) tail was not removed, broad bands were observed (Fig. 3B, lanes 1 and 3; Fig. 3C). The poly(A) lengths of *CGS1* mRNA prior to Met application distributed from 20 to 150 nt with a peak at 50–80 nt (Fig. 3B, lanes 1 and 2; Fig. 3C), whereas two peaks of poly(A) lengths, 10–30 nt and 140–150 nt, were observed 4 h after Met application (Fig. 3B, lanes 3 and 4; Fig. 3C).

The poly(A) tail of 50–80 nt before Met application can be attributed to that of full-length *CGS1* mRNA because the degradation intermediates were present only as a minor fraction (Fig. 1B). Four hours after Met application, the full-length *CGS1* mRNA was shown to carry a long poly(A) tail while the degradation intermediates carried a short poly(A) tail (Fig. 2B). Therefore, the poly(A) tails of 140–150 nt and 10–30 nt can be attributed to those of full-length and degradation intermediates, respectively, of *CGS1* mRNA.

***mto1* mutation abolishes the changes in poly(A) length after Met application** *mto1* mutations bear single amino acid sequence alterations within the MTO1 region, and abolish AdoMet-induced *CGS1* mRNA degradation (Chiba et al., 1999, 2003). In order to assess the involvement of MTO1 function in poly(A) length shift, total RNA prepared from *mto1-1* mutant calli before and after Met application was analyzed (Fig. 3B, lanes 5–8; Fig. 3D). *mto1-1* mutation is a Gly-84 to serine substitution and is one of the most severe alleles of *mto1* (Ominato et al., 2002).

The distribution of poly(A) lengths in *mto1-1* mutant *CGS1* mRNA before (0 h) Met application was essentially the same as that observed in wild-type calli before Met application with a peak at 50–80 nt. The distribution did not change appreciably 4 h after Met application. These results indicate that the changes in poly(A) length observed in wild-type *CGS1* mRNA after Met application was abolished in *mto1-1* mutant *CGS1* mRNA, and that the changes in poly(A) length are tightly linked to the negative feedback regulation of *CGS1* expression in response to AdoMet, for which the MTO1 region acts as the *cis*-element.

Time course of poly(A) length shift after Met application The poly(A) tail of the full-length *CGS1* mRNA shifted its length from 50–80 nt to 140–150 nt during the 4 h experimental period following Met application (Fig. 3). To clarify the temporal changes in poly(A) length after Met application, a time course analysis was conducted (Fig. 4). Following Met application, the 50–80 nt peak diminished by 0.5 h, and a poly(A) tail of 10–30 nt emerged. A poly(A) tail of 140–150 nt then gradually accumulated until 2 h after Met application (Fig. 4A, odd-numbered lanes; B). These results can be interpreted as demonstrating that the steady-state poly(A) tail of full-length *CGS1* mRNA under normal conditions is 50–80 nt, which is gradually replaced by the full-length *CGS1* mRNA with a poly(A) tail of 140–150 nt following Met application. On the other hand, no change in the length of the 3'-region of the *CGS1* mRNA body was detected in the samples from which the poly(A) tail was removed (Fig. 4A even-numbered lanes; C), providing further evidence that the position of the poly(A) addition site was not altered by Met application.

To evaluate the possibility that Met application may have had a general non-specific influence on poly(A) length, the temporal change in *UBQ5* mRNA poly(A) was analyzed after Met application (Fig. 4, B and D). The results show that *UBQ5* mRNA harbors a poly(A) tail of 30–40 nt, which did not appreciably change in response to Met application.

DISCUSSION

In this study, we showed that the poly(A) tail of full-length *CGS1* mRNA shifted in length from 50–80 nt to 140–150 nt after the induction of *CGS1* mRNA degradation by Met application. This result explains the increase in the total length of full-length *CGS1* mRNA after Met application (Fig. 1B). The potential contributions of other mechanisms, including alternate transcription start sites and/or poly(A) addition sites, or alternative splicing, were eliminated at least as major factor(s) (Figs. 2 and 4; Chiba et al., 2003).

We have reported previously that the half-life of *CGS1* mRNA in wild-type calli shifted from ~150 min to ~80 min after Met application (Lambein et al., 2003). This indicated that *CGS1* mRNA degradation is enhanced approximately twofold by feedback regulation. Nevertheless, the poly(A) tail of full-length *CGS1* mRNA increased its length in response to Met application. This appeared to be contradictory to the current understanding of poly(A) shortening, as mRNA that is destined for degradation is expected to have a shorter poly(A) tail (for reviews, see

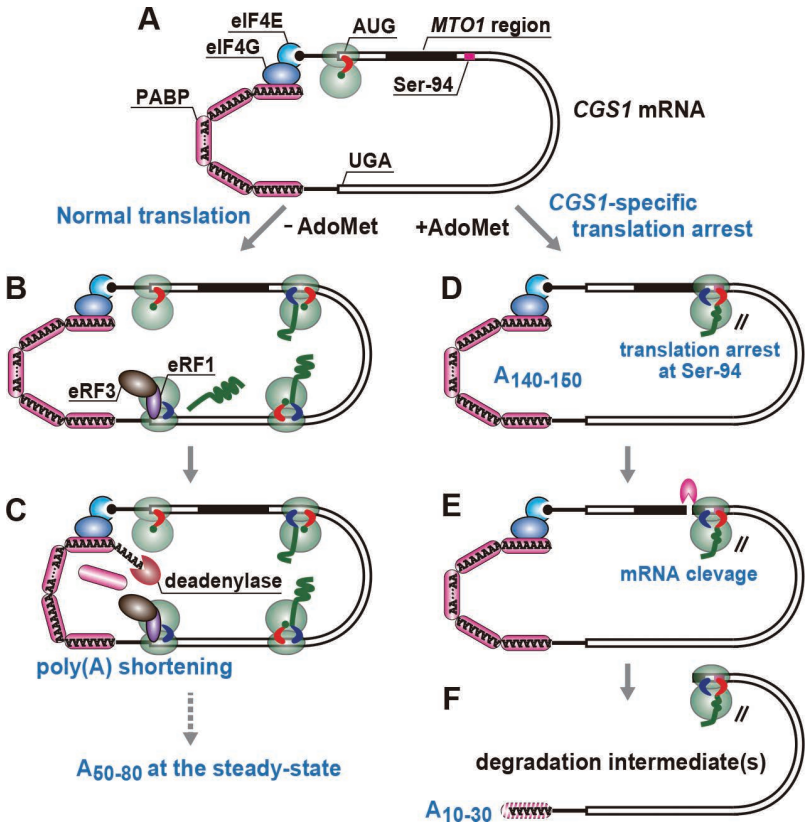

Fig. 5. Incorporation of differential poly(A) shortening in *CGS1* mRNA into the recent model for mRNA translation. The poly(A) lengths that were estimated in this study are indicated. (A) Actively translating mRNA exists in the form of a closed-loop structure through interactions of PABP and eIF4E-eIF4G cap-binding protein complex (Tarun and Sachs, 1996; Jacobson, 1996; Kahvejian et al., 2001). (B and C) Poly(A) shortening during normal translation. This mechanism applies to wild-type *CGS1* mRNA under a normal AdoMet condition, as well as to the *mto1* mutant *CGS1* mRNA, irrespective of the AdoMet conditions. (B) Upon termination of translation, eRF1-eRF3 complex releases the completed polypeptide, and the ribosomal subunits are recruited to a new round of translation through the interaction of eRF3 and PABP (Frolova et al., 1994; Zhouravleva et al., 1995; Uchida et al., 2002). (C) PABP occasionally recruits deadenylase to the poly(A) tail to shorten it (Hosoda et al., 2003; Funakoshi et al., 2007), resulting in a steady-state poly(A) tail of 50–80 nt in *CGS1* mRNA. (D–F) The fate of wild-type *CGS1* mRNA translated under excessive AdoMet levels. (D) Temporal translation arrest occurs at the Ser-94 codon immediately downstream of the *MTO1* region. The ribosome is arrested at the step of translocation and the CGS1 nascent peptide adopts a compact conformation (Onouchi et al., 2005; Onoue et al., 2011). Stacking of ribosome(s) behind the arrested ribosome (Haraguchi et al., 2008) is not shown here for simplicity. During translation arrest, the poly(A)-PABP interaction is expected to be static and hence the poly(A) tail is protected from deadenylation, resulting in a long poly(A) tail of 140–150 nt. (E) Translation arrest induces the *CGS1* mRNA degradation event near the 5'-edge of the arrested ribosome, mediated presumably by an endoribonuclease (magenta pacman) (Onouchi et al., 2005; Haraguchi et al., 2008), which is yet to be identified. The 5'-part of the degradation product(s) may be degraded rapidly and we have to date been unable to detect this product. (F) The 3'-part of the degradation product(s) has been identified as degradation intermediate(s) both *in vivo* and *in vitro* (Chiba et al., 2003). Rapid poly(A) shortening of the degradation intermediate occurs to give a 10–30 nt poly(A) tail. We have not determined whether or not the degradation intermediates bear PABP on it (indicated by a hatched PABP).

Parker and Song, 2004; Chiba and Green, 2009; Houseley and Tollervey, 2009). This raises the question as to how this poly(A) tail length shift in full-length *CGS1* mRNA can be explained when feedback regulation is induced and the mRNA is destined to be degraded.

The steady-state length of the poly(A) tail is determined by both the extent of polyadenylation in the nucleus, and the rate of poly(A) shortening in the cytosol. Polyadenylation also occurs in the cytosol (for review, see Richter, 2007). The fact that the poly(A) length of *UBQ5* mRNA did not change appreciably in response to Met application (Fig. 4, B and D) argues against the possibility that Met application has a general effect on nuclear and/or cytosolic polyadenylation. In addition, the fact that the poly(A) length of *mto1-1* mutant *CGS1* mRNA did not respond to Met application (Fig. 3) renders a *CGS1*-specific effect on nuclear and/or cytosolic polyadenylation unlikely.

An alternative scenario is that poly(A) shortening is inhibited or delayed in *CGS1* mRNA when it is targeted for AdoMet-induced degradation that is mediated by the MTO1 region. The above-mentioned results on *mto1-1* mutant *CGS1* mRNA (Fig. 3) provides strong support for this idea. We propose a model to account for the unusual poly(A) length behavior that takes into account the recent understanding of actively translating mRNA (Fig. 5). According to this understanding, mRNAs undergoing

active translation form a closed-loop structure in which the poly(A) tail and the 5'-cap are brought into close proximity by specific interactions between the poly(A)-binding protein (PABP) and the cap-binding protein complex, namely eukaryotic initiation factor (eIF) 4G and eIF4E (Tarun and Sachs, 1996; Jacobson, 1996; Kahvejian et al., 2001; Fig. 5A). During normal translation termination, eukaryotic release factor (eRF) 1 recognizes a termination codon and induces the release of a completed polypeptide in a way that is stimulated by eRF3 (Frolova et al., 1994; Zhouravleva et al., 1995). Poly(A) shortening is coupled with the termination of translation through the competitive interaction of eRF3 and deadenylase with PABP in yeast and mammalian cells. PABP exerts a pivotal role during the termination of translation in that PABP interacts, on the one hand, with the eRF1/eRF3 complex to facilitate another round of translation (Uchida et al., 2002), but, on the other hand, it can interact with the deadenylase complex to induce poly(A) shortening (Hosoda et al., 2003; Funakoshi et al., 2007) to give a steady-state poly(A) tail of 50–80 nt in *CGS1* mRNA under normal condition (Fig. 5, B and C).

In *CGS1* mRNA translated under conditions of excessive AdoMet, translation arrest at the Ser-94 codon prevents ribosomes from reaching the termination codon, thereby preventing poly(A) shortening to occur. Therefore, *CGS1* mRNA, with stalled ribosomes on it, is likely to be protected from poly(A) shortening, accounting for the appearance of a long poly(A) tail of 140–150 nt after Met application (Fig. 5D).

The degradation intermediates of *CGS1* mRNA had a poly(A) tail of 10–30 nt, suggesting that rapid poly(A) shortening occurs on degradation intermediates (Fig. 5, E and F). We do not yet know the mechanism for this rapid poly(A) shortening; however, we surmise that a disruption of the closed-loop structure is involved in this process. The degradation intermediates of *CGS1* mRNA are truncated at the 5'-region and are therefore in a form of decapped RNA. One might expect that decapped RNA would have a very short half-life since it would be readily degraded by 5'–3' exoribonuclease(s). However, these degradation intermediates are fairly stable and we have previously estimated their half-life to be 25–30 min in wild-type calli (Lambein et al., 2003). Since the 5'-ends of each of the *CGS1* mRNA degradation intermediates are positioned near the 5'-edge of the stalled ribosome (Onouchi et al., 2005; Haraguchi et al., 2008), it is possible that the stalled ribosome protects the degradation intermediates from 5'–3' exoribonuclease digestion (Fig. 5F). A similar situation is thought to occur in the *Bacillus subtilis ermCL* gene, which undergoes mRNA degradation coupled with antibiotic-induced translation arrest (Drider et al., 2002). Following a resumption of translation, the degradation intermediates may then enter the general mRNA degradation pathway as decapped mRNAs.

Changes in the poly(A) tail length in response to environmental or intra-cellular stimuli is not well studied in plants, with an exception of heat-shock protein mRNA (Osteryoung et al., 1993). As evidenced by the unique property of poly(A) shortening reported here, studies of the AdoMet-induced MTO1-dependent *CGS1* mRNA degradation will advance our understanding of the mechanisms that control mRNA stability, much of which is yet to be unveiled in plants.

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