Arabidopsis Cell-Free Extract, ACE, a New In Vitro Translation System Derived from Arabidopsis Callus Cultures

Katsunori Murota1, Yuka Hagiwara-Komoda1, Keisuke Komoda2,5, Hitoshi Onouchi1,3, Masayuki Ishikawa2 and Satoshi Naito1,4,*

1Division of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, Sapporo, 060-8589 Japan
2Plant-Microbe Interactions Research Unit, National Institute of Agrobiological Sciences, Tsukuba, 305-8602 Japan
3CREST, Japan Science and Technology Agency, Kawaguchi, 332-0012 Japan
4Division of Life Science, Graduate School of Life Science, Hokkaido University, Sapporo, 060-8589 Japan
5Present address: Faculty of Advanced Life Science, Hokkaido University, Sapporo, 060-0810 Japan.

*Corresponding author: E-mail, naito@abs.agr.hokudai.ac.jp; Fax, +81-11-706-4932

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The analysis of post-transcriptional regulatory mechanisms in plants has benefited greatly from the use of cell-free extract systems. Arabidopsis as a model system provides extensive genetic resources; however, to date a suitable cell-free translation system from Arabidopsis has not been available. In this study, we devised an Arabidopsis cell-free extract (ACE) to be used for in vitro translation studies. Protoplasts were prepared from callus cultures derived from Arabidopsis seedlings, and cell-free extracts were prepared after evacuation of the protoplasts by Percoll gradient centrifugation. The new ACE system exhibits translation activity comparable with that of the wheat germ extract system. We demonstrated that ACE prepared from the xrn4-5 exoribonuclease-deficient mutant of Arabidopsis, xrn4-5, exhibited increased stability of an uncapped mRNA as compared with that from wild-type Arabidopsis. We applied the ACE system to study post-transcriptional regulation of AtCGS1. AtCGS1 codes for cystathionine γ-synthase (CGS) that catalyzes the first committed step of methionine and S-adenosyl-L-methionine (AdoMet) biosynthesis in plants, and is feedback regulated by mRNA degradation coupled with translation elongation arrest. The ACE system was capable of reproducing translation elongation arrest and subsequent AtCGS1 mRNA degradation that are induced by AdoMet. The ACE system described here can be prepared in a month after seed sowing and will make it possible to study post-transcriptional regulation of plant genes while taking advantage of the genetics of Arabidopsis.

Keywords: Arabidopsis thaliana • AtXRN4 • In vitro translation • Mutation study • Post-transcriptional regulation • Wheat germ extract.

Abbreviations: ACE, Arabidopsis cell-free extract; AdoMet, S-adenosyl-L-methionine; BY-2, Bright Yellow-2; BYL, BY-2 lysate; CGS, cystathionine γ-synthase; GST, glutathione S-transferase; LUC, firefly luciferase; PIPES, piperazine-1,4-bis(2-ethanesulfonic acid); RLUC, Renilla reniformis luciferase; RRL, rabbit reticulocyte lysate; WGE, wheat germ extract.

Introduction

In order to understand the various phenomena that occur in living cells, it is important to provide molecular level information on the mechanisms associated with the respective phenomenon. A cell-free translation system is one of the useful tools to investigate detailed molecular mechanisms in vitro. *Escherichia coli* cell extract, wheat germ extract (WGE) and rabbit reticulocyte lysate (RRL) are the in vitro translation systems frequently employed and are also commercially available (Oliver et al. 1998, Hino et al. 2008, Nishiyama et al. 2010). In addition, several systems have been developed using various organisms including human HeLa cells (Withrell 2001), yeast (*Saccharomyces cerevisiae*) and insect (*Neurospora crassa*) (Wang and Sachs 1997) cells. The use of mutant cells when preparing extracts, if applicable, enables these systems to be modified when needed. For example, a cell-free extract prepared from a protease-deficient *E. coli* mutant was used to improve protein productivity (Ali et al. 2005).

We have previously developed a cell-free translation system derived from tobacco Bright Yellow-2 (BY-2) cells (Komoda et al. 2004). The BY-2 lysate, termed BYL, is capable of supporting the translation and subsequent replication of genomic RNAs of plant positive-strand RNA viruses (Komoda et al. 2004, Shibashi et al. 2007, Iwakawa et al. 2007, Gursinsky et al. 2009, Shibashi et al. 2009), and the formation of RNA-induced silencing complexes (Iki et al. 2010). However, since BY-2 is derived from an amphidiploid tobacco (*Nicotiana tabacum*) for which the genome sequence has not been fully determined and a collection of T-DNA tagging lines is not available, it


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is often difficult to prepare BYL lacking a specific gene function. A new system that overcomes these limitations was therefore required for molecular genetic analyses.

We have previously applied WGE and RRL to study post-transcriptional regulation of AtCGS1 (gene ID At3g01120) that codes for cystathionine γ-synthase (CGS; EC 2.5.1.48), the first committed enzyme of methionine and S-adenosyl-L-methionine (AdoMet) biosynthesis in plants (Matthews 1999). Expression of the AtCGS1 gene is down-regulated at the step of mRNA degradation in response to AdoMet (Chiba et al. 1999, Chiba et al. 2003). This post-transcriptional regulation is inhibited by a translation inhibitor, suggesting that the regulation takes place during translation of AtCGS1 mRNA (Lambein et al. 2003). The use of WGE enabled this regulation to be recapitulated, and the same mRNA degradation intermediates that are found in vivo have been detected in WGE (Chiba et al. 2003, Onouchi et al. 2005, Haraguchi et al. 2008). In RRL, however, AdoMet-induced translation arrest was recapitulated but the AtCGS1 mRNA degradation intermediate was not detected (Onouchi et al. 2008). These results suggested that plant-specific trans-acting factors are required in the regulation. However, the current system using WGE does not allow genetics to be applied to the in vitro translation studies. A cell-free translation system derived from Arabidopsis will become a powerful tool by overcoming the limitations of both WGE and BYL, and enabling in vitro studies to take advantage of genetics.

We report here the development of a cell-free translation system derived from liquid callus cultures of Arabidopsis. The obtained Arabidopsis cell-free extract (ACE) was characterized by comparison with WGE. Applicability of genetics to the ACE system was exemplified by analyzing degradation of uncapped mRNA in ACE prepared from the Arabidopsis xrn4-5 mutant line. AtXRN4 has been suggested to be a cytosolic 5′–3′ exoribonuclease acting in the degradation of mRNA (Souret et al. 2004, Rymarquis et al. 2011). We also show that the autoregulation of AtCGS1 mRNA stability coupled with translation arrest can be recapitulated in ACE.

Results and Discussion

Liquid callus culture and preparation of ACE

To obtain ACE, dedifferentiated calli of Arabidopsis were first prepared. One-week-old seedlings of Arabidopsis were finely minced with a razor blade under aseptic conditions (Fig. 1A, B) and then cultured in a suspension medium with constant agitation (Fig. 1C). Following a 21 d incubation during which...
culture medium was refreshed three times, protoplasts were isolated from the calli. Calli were cultured in the dark (Fig. 1D) to avoid development of chloroplasts, since this increases the yield of the evacuolated protoplasts compared with that from calli with developed chloroplasts.

In most plant cells, lytic vacuoles that contain hydrolytic enzymes such as proteases and RNases occupy most of the intracellular space. To remove the vacuoles from Arabidopsis protoplasts, the method developed by Sonobe (1996) and modified for BYL (Komoda et al. 2004, Ishibashi et al. 2007, Komoda et al. 2007, Ishibashi et al. 2006) was applied. In this method, low-buoyant density vacuoles are detached from the protoplasts, yielding high-buoyant density evacuated protoplasts. To obtain evacuolated protoplasts, protoplast-containing solution was layered on top of a Percoll gradient. After centrifugation, evacuated protoplasts were concentrated at the interface between 35% and 55% Percoll layers (Fig. 1E–H). The evacuated protoplasts were collected, washed and homogenized. Nuclei and non-disrupted cells were then removed by centrifugation. The supernatant was gel filtrated to remove endogenous low molecular weight factors that might affect the efficiency of translation, such as AdoMet, polyamines and amino acids, to obtain ACE. A detailed protocol to prepare ACE is described in the Materials and Methods.

**In vitro translation activity of ACE**

To evaluate the translation activity of ACE, in vitro synthesized 5’-capped mRNA encoding *Renilla reniformis* luciferase (RLUC) was translated in ACE and WGE for 120 min. Unless otherwise stated, 5’ cap analog, m7G(5’)-ppp(5’)-G, was included in the in vitro transcription reaction to synthesize mRNA with a 5’ cap structure in this study. RLUC activity analysis revealed that the translation activity of ACE was comparable with that of WGE (Fig. 1I). Measurements of RLUC activities in four different ACE preparations showed a relative SD of ±17% (Supplementary Table S1). On the other hand, protoplast extract prepared without the evacuation step showed essentially no translation activity (Fig. 1I).

To determine the optimum concentration of input mRNA that provides a maximum amount of translation product, various concentrations of in vitro synthesized glutathione S-transferase (GST) mRNA were translated in ACE or WGE. In ACE, the amount of synthesized GST protein increased with the amount of input mRNA between concentrations of 1 and 50 fmol μl⁻¹. Higher mRNA concentrations did not appreciably increase the amount of the translation product, indicating that 50 fmol μl⁻¹ input mRNA was sufficient to achieve a maximum level of protein synthesis (Fig. 2A, upper panel). In WGE, addition of 20–50 fmol μl⁻¹ mRNA achieved a maximum level of protein product accumulation (Fig. 2A, lower panel).

The temporal pattern of protein product accumulation was next determined by adding 50 fmol μl⁻¹ of GST mRNA to ACE or WGE, and incubating the translation reaction mixtures for various time periods. While the protein product accumulation increased until 120–180 min in WGE (Fig. 2B, lower panel), the protein synthesis in ACE appeared to reach a plateau after a 60–90 min incubation (Fig. 2B, upper panel).

**Stability of uncapped mRNA in ACE**

In commercially obtained WGE, input RNAs do not need to be 5’ capped for protein synthesis. To investigate the 5’ cap structure dependency of mRNA to be used as a translation template in the ACE system, in vitro synthesized capped and uncapped *Luc* mRNA coding for firefly luciferase (LUC) were translated in ACE and WGE. As an internal control for the translation reaction, capped *Rluc* mRNA was co-translated, and the LUC activities relative to RLUC activities were compared. In ACE translating uncapped *Luc* mRNA, the relative LUC activity was decreased to approximately 3% of that in ACE translating capped *Luc* mRNA (Fig. 3A). On the other hand, the relative LUC activity in WGE translating uncapped mRNA was approximately 60% of that in WGE translating capped mRNA (Fig. 3A), confirming that commercially obtained WGE shows only weak cap dependency.

To investigate further the cap dependency of reporter activity in ACE, *Luc* mRNA with or without the 5’ cap was translated in ACE or WGE. Samples were taken at various time points...
and the integrity of the input mRNA was analyzed. As shown in Fig. 3B, when capped mRNA was translated, 30–40% of the input RNA was still detectable at the full-length position after 120 min of translation reaction, whereas >20% of the input RNA persisted at the full-length position in WGE. The result shows that, unlike in WGE, the uncapped mRNA is rapidly degraded in ACE as in vivo. Uncapped mRNA has been shown also to be unstable in BYL (Sarawaneeyaruk et al. 2009).

mRNA stability analysis in ACE derived from the xrn4-5 mutant Arabidopsis

AtXRN4 is a functional homolog of yeast Xrn1p, a 5′→3′ exoribonuclease, and is suggested to be involved in degradation of mRNA in the cytosol (Kastenmayer and Green 2000, Souret et al. 2004, Rymarquis et al. 2011; for a review, see Chiba and Green 2009). XRN4 in plants functions in down-regulation of uncapped viral RNA accumulation (Cheng et al. 2007, Jaag et al. 2009), and is also reported to be a negative regulator of transgene-induced gene silencing (Gazzani et al. 2004). AtXRN4 has been identified as the causal gene for the ethylene-insensitive mutant of Arabidopsis, ein5 (Olmedo et al. 2006, Potuschak et al. 2006).

As we showed in Fig. 3B, uncapped mRNA was rapidly degraded in the ACE system. We investigated whether the stability of uncapped Luc mRNA was increased in ACE derived from the AloxR4 T-DNA insertion line, xrn4-5 (Souret et al. 2004). To evaluate the stability of input RNA separately from the translation reaction, a translation inhibitor, puromycin (Carrasco et al. 1976), was added to the ACE reaction mixture in this experiment, and degradation of input mRNA during incubation in ACE was analyzed (Fig. 4A–C). In ACE prepared from wild-type Arabidopsis, approximately 90% of uncapped Luc mRNA was degraded within 10 min incubation (Fig. 4A, B). On the other hand, in ACE prepared from the xrn4-5 mutant, >30% of uncapped Luc mRNA was still detectable at the full-length position after 10 min incubation (Fig. 4A, C). This result suggests that AtXRN4 participates in degradation of uncapped Luc mRNA in ACE. However, even in the absence of AtXRN4, suppression of uncapped mRNA degradation was not complete and, after 30 min incubation, approximately 80% of uncapped Luc mRNA was degraded, suggesting that ACE prepared from the xrn4-5 mutant contains other RNase(s) that degrade uncapped mRNA.

To determine the effect of the xrn4-5 mutation on uncapped mRNA translation, capped and uncapped Luc mRNAs were translated in wild-type or xrn4-5 mutant ACE. Capped RLuc mRNA was co-translated and used as an internal control. As shown in Fig. 4D, when uncapped Luc mRNA was translated in xrn4-5 mutant ACE, the relative LUC activity was 3- to 4-fold higher than that in wild-type ACE, which is apparently consistent with the stabilization of uncapped mRNA in xrn4-5 mutant ACE.

Post-transcriptional regulation of AtCGS1 mRNA in response to AdoMet: an application of the ACE system

To test the applicability of the ACE system in molecular studies of post-transcriptional regulation, we next examined whether
the regulation of AtCGS1 gene expression is recapitulated in ACE. Expression of the AtCGS1 gene is feedback regulated at the step of its mRNA degradation (Chiba et al. 1999, Chiba et al. 2003). The exon 1 coding sequence (183 amino acids) of AtCGS1 is necessary and sufficient for this regulation (Suzuki et al. 2001), and a short stretch of amino acid sequence termed the MTO1 region acts as a cis-element (Ominato et al. 2002). mto1 mutations of Arabidopsis are point mutations that alter the amino acid sequence of the MTO1 region and disrupt the feedback regulation, resulting in an overaccumulation of soluble methionine (Inaba et al. 1994, Chiba et al. 1999, Ominato et al. 2002). This post-transcriptional regulation is recapitulated in an in vitro translation system of WGE (Chiba et al. 2003). Studies using WGE showed that AdoMet induces temporal translation elongation arrest at the Ser94 codon located immediately downstream of the MTO1 region and, as a result, peptidyl-tRNA\textsubscript{ser} accumulates (Onouchi et al. 2005). The AtCGS1 mRNA degradation produces 5'-truncated AtCGS1 mRNA species as degradation intermediates (Chiba et al. 2003, Haraguchi et al. 2008).

We examined whether the regulation of AtCGS1 exon 1 expression is observed in ACE as was the case in WGE using RNAs containing AtCGS1 exon 1 fused in-frame to a Luc gene [Ex1(WT):Luc] or its mto1-1 mutant derivative [Ex1(mto1-1):Luc] (Fig. 5A). The LUC activity was repressed when Ex1(WT):Luc was translated in the presence of AdoMet, whereas such repression of the LUC activity was not observed with Ex1(mto1-1):Luc mRNA (Fig. 5B). These results are consistent with the results shown with WGE in this study (Fig. 5C), and with a previous report in both WGE and in vivo (Chiba et al. 2003).

In our previous report, we showed that the ribosome stalls at the step of translocation in response to AdoMet using the WGE in vitro system (Onouchi et al. 2005). However, direct evidence of translation arrest has not yet been detected in Arabidopsis. We therefore tested whether translation arrest in response to AdoMet is reproduced in ACE. RNAs harboring GST fused in-frame with wild-type AtCGS1 exon 1 [GST:Ex1(WT)] or its mto1-1 mutant derivative [GST:Ex1(mto1-1)] (Fig. 6A) were translated in ACE in the presence or absence of AdoMet for 30 min. Translation products were analyzed by immunoblot analysis using anti-GST antibody (Fig. 6B). In the presence of AdoMet, the accumulation of a full-length protein product with a molecular mass of 45 kDa (Fig. 6B, open arrowhead) was reduced, while 55 kDa bands appeared in ACE as well as in WGE. The 55 kDa products correspond to the AdoMet-induced translation arrest products that are peptidyl-tRNA\textsubscript{ser} (Fig. 6B, filled arrowhead with a cloverleaf) (Onouchi et al. 2005, Haraguchi et al. 2008, Onoue et al. 2011), suggesting that the translation arrest takes place in ACE.

In WGE, AtCGS1 mRNA is degraded following translation elongation arrest, and a ladder of 5'-truncated mRNA species, with their 5' end position separated by about 30 nucleotides, is accumulated in response to AdoMet as mRNA degradation intermediates (Chiba et al. 2003, Onouchi et al. 2005,
Upon translation arrest, translating ribosomes are stacked behind the initially arrested ribosome, and mRNA degradation events occur near the 5′ edge of these stacked ribosomes (Haraguchi et al. 2008). The size of 30 nucleotides matches the region of mRNA that is covered by a ribosome (Wolin and Walter 1988).

We tested whether the 5′-truncated RNAs are also produced in ACE in the presence of AdoMet. To detect mRNA degradation intermediates in ACE, GST:Ex1(WT) and GST:Ex1(mto1-1) were translated for 30 min at 25°C in the presence (+) or absence (−) of 1 mM AdoMet in ACE (left) or WGE (right). Translation products were analyzed by immunoblot analysis using an anti-GST antibody. The filled arrowhead with a cloverleaf marks the position of peptidyl-tRNA (Pep-tRNA, approximately 55 kDa) and the open arrowhead marks the position of the full-length product (Full-length, 45 kDa). The two bands in the peptidyl-tRNA (indicated by a bracket) correspond to initially and secondarily stalled ribosomes (see below in C). A representative result of triplicate experiments is shown.

Fig. 5 AdoMet-induced down-regulation of reporter activity in mRNA carrying AtCGS1 exon 1. (A) Schematic representation of Ex1(WT):Luc mRNA and Ex1(mto1-1):Luc mRNA that carries the AtCGS1 exon 1 coding sequence (Ex1) fused in-frame to Luc DNA. The approximate position and amino acid sequence change in the mto1-1 mutation are indicated. (B and C) Capped Ex1(WT):Luc mRNA (filled squares) and Ex1(mto1-1):Luc mRNA (open squares) were translated for 120 min at 25°C using ACE (B) or WGE (C), in the presence of various concentrations of AdoMet as indicated. The relative reporter activity was normalized with the RLUC activity. Normalized LUC activity relative to that in samples without AdoMet was calculated. The averages ± SD of three experiments are shown.

Fig. 6 AdoMet-induced translation elongation arrest of mRNA carrying AtCGS1 exon 1. (A) Schematic representation of GST:Ex1(WT) mRNA and GST:Ex1(mto1-1) mRNA that carries a GST tag sequence at the N-terminus of AtCGS1 exon 1 (Ex1). The approximate position and amino acid sequence change in the mto1-1 mutation are indicated. Amino acid numbers encoded by AtCGS1 exon 1 are indicated above the boxes. (B) Capped GST:Ex1(WT) or GST:Ex1(mto1-1) mRNA were translated for 30 min at 25°C in the presence (+) or absence (−) of 1 mM AdoMet in ACE (left) or WGE (right). Translation products were analyzed by immunoblot analysis using an anti-GST antibody. The filled arrowhead with a cloverleaf marks the position of peptidyl-tRNA (Pep-tRNA, approximately 55 kDa) and the open arrowhead marks the position of the full-length product (Full-length, 45 kDa). The two bands in the peptidyl-tRNA (indicated by a bracket) correspond to initially and secondarily stalled ribosomes (see below in C). A representative result of triplicate experiments is shown. (C) Schematic illustration of the peptidyl-tRNA produced by the AdoMet-induced translation arrest (Onouchi et al. 2005, Haraguchi et al. 2008). In the presence of AdoMet, the ribosome stalls at the Ser94 codon, and a second ribosome stacks behind the initially stalled ribosome, producing partial translation products of peptidyl-tRNAs. The tRNA moiety in the peptidyl-tRNAs gives an approximately 20 kDa shift, depending on the tRNA species, in PAGE. Note that commercially available WGE contains an added bovine tRNA preparation while ACE uses endogenous tRNA contained in the extract, which would account for the small difference in the peptidyl-tRNA band positions between ACE and WGE in B. The full-length product gives an approximately 45 kDa (26 kDa GST tag + 183 amino acids of AtCGS1 exon 1) band, whereas the peptidyl-tRNASer of the initially stalled ribosome gives an approximately 57 kDa (26 kDa GST tag + 94 amino acids of AtCGS1 exon 1 + tRNASer) band. The exact position of the secondarily stalled ribosome is not determined.
RNAs were translated in the presence of 1 mM AdoMet, followed by primer extension analysis. When GST:Ex1(WT) mRNA was translated, a series of 5'-truncated RNA species were detected in both ACE and WGE (Fig. 7). When GST:Ex1(mto1-1) mRNA was translated, these truncated RNAs were not observed in ACE or WGE. While we detected at least four 5'-truncated RNA species in WGE, we only detected two of these in ACE (Fig. 7). Although the reason for this difference is not clear, it is intriguing to note that we also detected these two bands in vivo (Chiba et al. 2003). These results demonstrated that AtCGS1 mRNA degradation coupled with translation arrest was recapitulated in ACE.

The use of the ACE system in molecular genetics
Although WGE is a convenient system to produce proteins in vitro, the potential application of in vitro translation systems could go beyond simply obtaining a protein product. In order to use WGE to identify factor(s) that are involved in a biological phenomenon, inactivation or modification of the relevant factor(s) would be required to provide important information in elucidating the relevant molecular mechanism. However, inactivation of the factor(s) by the addition of an antibody or inhibitors is almost the sole choice in WGE. In the case of BYL, an RNA interference strategy is applicable to inactivate the relevant factor(s) in BY-2 cells; however, this approach is time consuming and it is difficult to achieve full inactivation of the target gene. In contrast, ACE can be prepared in a month not only from the wild type but also from mutant lines of Arabidopsis. As exemplified by the analysis of uncapped mRNA degradation in the xrn4-5 mutant, ACE, as well as post-transcriptional regulation of AtCGS1 in wild-type ACE, reproduction of intracellular mechanisms in vitro is now possible using ACE. The ACE system reported here will provide us with a new strategy for the in vitro studies by applying the strengths of both forward and reverse genetics of Arabidopsis.

Materials and Methods

Isolation of protoplasts from liquid callus cultures of Arabidopsis
Preparation of Arabidopsis liquid callus culture and isolation of protoplasts were performed essentially according to Guzman and Ecker (1988) as modified by Ishikawa et al. (1993). In brief, Arabidopsis seeds were surface-sterilized using a solution containing 50% antiformine and 0.15% Tween-20 for 5 min, and washed 10 times with sterilized distilled water. Sterilized seeds were aseptically sown on MS plate medium [4.6 g l⁻¹ Murashige and Skoog salts (Wako Pure Chemicals), 0.5 g l⁻¹ MES, 10 g l⁻¹ sucrose, 1 mg l⁻¹ thiamine-HCl, 0.5 mg l⁻¹ pyridoxine, 0.5 mg l⁻¹ nicotinic acid, 100 mg l⁻¹ myo-inositol and 0.8% agar (pH 5.7)] and treated at 4°C for 2 d in the dark. Seeds were then incubated at 22°C for 1 week under a constant light condition for germination.

Fig. 7 Detection of the AdoMet-induced mRNA degradation intermediate in AtCGS1 exon 1. Capped GST:Ex1(WT) mRNA or GST:Ex1(mto1-1) mRNA were translated for 120 min in the presence (+) or absence (−) of 1 mM AdoMet in ACE or WGE. Poly(A) RNA was extracted and subjected to primer extension analysis using a 5'-³²P-labeled TO4L primer (Chiba et al. 2003). Lanes A, C, G and T indicate the sequence ladder synthesized using the same primer. The larger arrowheads indicate the positions of the 5'-truncated RNA detected both in ACE and in WGE, while the smaller arrowheads indicate the positions of the 5'-truncated RNA detected only in WGE. The position of the full-length extension product is marked with an open arrowhead. A representative result of triplicate experiments is shown. The contrast of the original data was enhanced to improve visibility of the 5'-truncated RNA species.
For the induction of calli, 100–150 seedlings were minced with a razor blade until they become <1 mm pieces on filter paper (No. 2, Advantec) soaked in RM28 medium [4.6 g l<sup>-1</sup> Murashige and Skoog salts, 0.1 M sucrose, 1 mg l<sup>-1</sup> thiamine-HCl, 0.5 mg l<sup>-1</sup> pyridoxine, 0.5 mg l<sup>-1</sup> nicotinic acid, 100 mg l<sup>-1</sup> myo-inositol, 0.2 mg l<sup>-1</sup> 2,4-D, 50 mg l<sup>-1</sup> kinetin and 3 mM MES (pH 5.8)] under aseptic conditions. The minced seedlings were transferred to a 200 ml Erlenmeyer flask containing 50 ml of RM28 medium and incubated at 23 °C in the dark with constant shaking (100 r.p.m., orbital diameter 5 cm). The medium was exchanged once every 6 d.

On the third day after the third medium transfer (30th day after seed sowing), the callus cultures were used to isolate protoplasts. Subsequent manipulations were not necessarily performed under aseptic conditions. The calli were collected and washed with 0.6 M mannitol, followed by incubation with PIM which consists of RM28 medium with 5 mM CaCl<sub>2</sub>, 0.6 M mannitol, 1% cellulase Onozuka RS (Yakult Pharmaceutical Industry) and 0.2% Pectolyase Y23 (Kyowa Chemical Products). The incubation was performed at 28 °C for 3 h in a 200 ml Erlenmeyer flask with gentle agitation. After incubation, the protoplast suspension was filtered through a 75 μm mesh to remove calli. Protoplasts were collected by centrifugation at 100 × g for 5 min, and washed twice with ice-cold 0.6 M mannitol.

### Preparation of Arabidopsis cell extract from evacuated protoplast

To obtain evacuated protoplasts, approximately 1 ml of protoplasts were mixed with 2 ml of 5% Percoll solution. The protoplast solution was overlaid on 9 ml of a 10–35% (v/v) Percoll linear gradient layered on 3 ml of 55% (v/v) Percoll solution was overlaid on 9 ml of a 10–35% (v/v) Percoll density gradient in an open-top centrifuge tube (#361707, Beckman Coulter). All Percoll solutions contained 0.7 M mannitol, 0.2 M glycine, and 55% Percoll layers. The evacuated protoplasts were collected by centrifugation at 100,000 × g for 5 min, and washed twice with ice-cold 0.6 M mannitol.

Plasmids used for in vitro transcription

Plasmids pMI21(WT) and pMI21(mto1-1), which carry Ex1(WT):Luc and Ex1(mto1-1):Luc DNA, respectively, in the pSP64 poly(A) vector (Promega) were described previously (Chiba et al. 2003). Plasmids pYF2 and pYF3 carry GST:Ex1(WT) and GST:Ex1(mto1-1) whose XbaI site immediately 5′ of the GST-coding region was disrupted (Haraguchi et al. 2008).

Plasmid pSY013 carries the Luc gene in the pSP64 poly(A) vector. Luc DNA was excised from pMI21(WT) by digestion with HindIII and SacI. Plasmid pKM4 carries a GST-coding sequence in the pSP64 poly(A) vector. pKM4 was constructed from pYF2 DNA that carries GST:Ex1(WT) DNA by eliminating the AICGST1 exon 1 sequence. The GST-coding sequence and the vector region in pYF2 were PCR amplified using the primers, 5′-TAGTCTTTGAGCCGAGGCTC-3′ and 5′-ATCCGATTTTGGAGGATGCT-3′, and circularized after digesting the template DNA by DpnI (Liu and Naismith 2008) and phosphorylating the 5′ ends by T4 polynucleotide kinase. Plasmid pMI27 carrying the RLuc gene in the pSP64 poly(A) vector has been described (Chiba et al. 2003).

The integrity of PCR-amplified sequences was verified by sequence analysis.

### In vitro transcription

DNA templates in pSP64 poly(A) vector (Promega) were linearized with EcoRI digestion (Chiba et al. 2003). Capped mRNA was transcribed in vitro in the presence of a cap analog m<sup>7</sup>G[5′]ppp[5′]G using the AmpliCap SP6 high yield message maker kit (Epigen Biotechnology). Uncapped mRNA was transcribed in vitro using a CUGA6 in vitro transcription kit (Nippon Genetech). Poly(A) RNA was purified as described (Chiba et al. 2003).

<sup>32</sup>P-Labeled Luc mRNA was transcribed using an AmpliCap SP6 in vitro transcription high yield message marker kit, with the following modifications. The reaction mixture contained 4.7 mM ATP, 4.7 mM UTP, 0.94 mM GTP, 0.02 mM CTP, 3.75 mM cap analog, m<sup>7</sup>G[5′]ppp[5′]G (Promega) and 740 kBq of [α-<sup>32</sup>P]CTP (Hungarian Academy of Sciences, 111 TBq mmol<sup>−1</sup>) substituted for the Cap/NTP mix. Uncapped Luc mRNA was transcribed without the cap analog.

### In vitro translation

The in vitro translation reaction mixture for ACE contained 0.75 mM ATP, 0.1 mM GTP, 20 mM creatine phosphate, 40 μM cysteine, 40 μM leucine, 80 μM of the other 18 amino acids, 500 μM spermidine, 0.2 μg μl<sup>−1</sup> creatine kinase and 0.8 μl<sup>−1</sup> RNase inhibitor (Promega). In vitro synthesized mRNA was added to the reaction mixture and the translation reaction was performed at 25 °C. The in vitro translation reaction was terminated by 50-fold dilution of the reaction mixture with ice-cold water for reporter assay, by mixing with 9 x volume of gel sample buffer (2% SDS, 0.1 M dithiothreitol, 50 mM Tris–HCl pH 6.8 and 10% glycerol) followed by boiling...
for 3 min for protein product analyses, or by directly transferring to RNA extraction for RNA analyses.

In vitro translation reactions using WGE (Promega) were carried out as described (Chiba et al. 2003), except that for experiments other than when AtCGS1 exon 1 was used, the final concentrations of amino acids were 40 μM cysteine, 40 μM leucine and 80 μM for the other 18 amino acids.

**Product analyses**

RLUC activity was assayed by using a *Renilla* Luciferase Assay System kit (Promega). For dual reporter analysis, LUC and RLUC activities were assayed by using a double-luciferase assay system (Promega). For RNA stability analyses of fluorescently labeled RNA, 32P-labeled radiolabeled RNA was used in a 20 μl reaction mixture, and protein products were separated on a NuPAGE 4–12% Bis–Tris gel (Invitrogen). Detection of GST by anti-GST antibody was carried out as described (Onouchi et al. 2005).

**RNA analyses**

For RNA stability analyses of *Luc* mRNA, RNAs extracted from the ACE or WGE reaction mixture were subjected to Northern hybridization or direct size separation of radiolabeled mRNA. For Northern hybridization, extracted RNA was separated on a 5% polyacrylamide gel containing 1× Denaturing Buffer and transferred to a Hybond N+ membrane (GE Healthcare). The membrane was hybridized with a 32P-labeled probe, which was prepared by PCR amplification of pSY013 DNA, using the primers 5′-ACCGCTGG-3′ and 5′-TTCACTGCATACGACGATTCTG-3′. The membrane was washed and exposed to X-ray film.

For reporter assays, 50 fmol μl−1 GST or GST:Ex1 mRNA was used for the translation reaction in a 20 μl reaction mixture, and protein products were separated on a NuPAGE 4–12% Bis–Tris gel (Invitrogen). Detection of GST by anti-GST antibody was carried out as described (Onouchi et al. 2005).

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