A Dual Upstream Open Reading Frame-based Autoregulatory Circuit Controlling Polyamine-responsive Translation*

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A novel form of translational regulation is described for the key polyamine biosynthetic enzyme 3-adenosylmethionine decarboxylase (AdoMetDC). Plant AdoMetDC mRNA 5' leaders contain two highly conserved overlapping upstream open reading frames (uORFs): the 5' tiny and 3' small uORFs. We demonstrate that the small uORF-encoded peptide is responsible for constitutively repressing downstream translation of the AdoMetDC proenzyme ORF in the absence of increased polyamine levels. This first example of a sequence-dependent uORF to be described in plants is also functional in Saccharomyces cerevisiae. The tiny uORF is required for normal polyamine-responsive AdoMetDC mRNA translation, and we propose that this is achieved by control of ribosomal recognition of the occluded small uORF, either by ribosomal leaky scanning or by programmed −1 frameshifting. *In vitro* expression demonstrated that both the tiny and the small uORFs are translated. This tiny/small uORF configuration is highly conserved from moss to Arabidopsis thaliana, and a more diverged tiny/small uORF arrangement is found in the AdoMetDC mRNA 5' leader of the single-celled green alga Chlamydomonas reinhardtii, indicating an ancient origin for the uORFs.

Polymamines are small, organic polycations found in all eukaryotic and most prokaryotic cells (1) and are involved in core cellular processes such as chromatin organization, mRNA translation, ribosome biogenesis, cell proliferation, and apoptosis (2). In bovine lymphocytes ~60% of spermidine (spd)* and spermine (spm) cellular content is bound to RNA (3). The precursor polyamine, putrescine (1,4-diaminobutane), is converted into spd by the addition of an aminopropyl group donated by decarboxylated 3-adenosylmethionine (AdoMet). Spm is formed from spd by symmetrical addition of another aminopropyl group. The formation of decarboxylated AdoMet by AdoMet decarboxylase (AdoMetDC; EC 4.1.1.50) is therefore a key step in polyamine biosynthesis.

Translational control allows rapid changes to the proteome that do not require slower transcriptional responses (4). Mammalian AdoMetDC mRNA is translationally regulated by polyamines (5) through a single uORF. The role of uORFs in translational regulation is recognized as an important component of gene expression control (6, 7). There are two characterized types of uORF: sequence-independent uORFs, where uORF recognition, uORF termination efficiency, intercistronic distance, and sequence affect reinitiation efficiency at the downstream ORF (but the uORF-encoded peptide is not important), and sequence-dependent uORFs, where the nascent uORF peptide causes ribosome stalling during translational elongation or termination. The distinct mechanisms of these two types of uORFs have been demonstrated physically (8).

The best studied examples of sequence-dependent uORFs are in the arginine-responsive Saccharomyces cerevisiae CPA1 (9) and Neurospora crassa arg-2 (10) mRNAs encoding carbamoyl-phosphate synthetase, the cytomegalovirus gpUL4 mRNA (11), and the polyamine-responsive mammalian AdoMetDC mRNA (12). Sequence-dependent uORFs stall ribosomes at translation termination (13) or during elongation of the uORF peptide (14). Until now, no sequence-dependent uORF has been identified experimentally in plants.

The mammalian AdoMetDC uORF encodes the hexapeptide MAGDIS and is located 14 nucleotides downstream of the 5' cap. MAGDIS-mediated translational regulation of the AdoMetDC mRNA depends on cell type (15) and cellular polyamine content (16). Increased spd levels cause ribosome stalling at the termination codon as detected by toe printing and expression analysis in a gel-filtered rabbit reticulocyte lysate system (17, 18).

Plant AdoMetDC mRNAs possess long 5' leader sequences of at least 500 nucleotides containing a pair of uORFs that overlap by one nucleotide (19). The 5' tiny uORF and the overlapping 3' small uORF consist of 3–4 and 48–54 codons, respectively, and their highly conserved arrangement predates the origin of flowering plants (19). Previously, we demonstrated that the plant AdoMetDC mRNA is post-transcriptionally regulated by polyamines and that the small uORF represses downstream translation under normal growth conditions (20). Removal or truncation of the small uORF abolishes translational control, disrupts polyamine levels, severely perturbs growth of transgenic plants (20), and results in the depletion of chloroplast RNA-binding proteins (21). Here we show that the tiny and small uORFs are essential for translational regulation by polyamines, acting as a dual component mechanism; the sequence-independent tiny uORF is required for normal levels of polyamine responsiveness, and the sequence-dependent small uORF is required for downstream translational repression. Our results are consistent with the proposal that polyamine-responsive translational repression of the AdoMetDC mRNA is due to a ribosomal switch from the noninhibitory tiny uORF to the inhibitory small uORF. The small uORF peptide is the first sequence-dependent uORF identified in plants, and it is likely to target a highly conserved component of the translational machinery, because the Arabidopsis small uORF is also...
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repressive in a sequence-dependent manner in *S. cerevisiae*. In addition, we found that the *AdoMetDC* mRNA from the single-celled green alga *Chlamydomonas reinhardtii* contains a more diverged tiny/small uORF arrangement containing a perfectly preserved intron position in the small uORF, suggesting that the plant *AdoMetDC* mRNA uORFs represent an ancient mechanism for translational regulation in response to polyamines.

**EXPERIMENTAL PROCEDURES**

*Site-directed Mutagenesis*—Site-directed mutants of the *AdoMetDC1* cDNA were produced using the Chameleon double-stranded mutagenesis kit (Stratagene, La Jolla, CA), following the manufacturer’s instructions. Mutants were constructed using the *SAMDC1* plasmid (which contains the wild type *AdoMetDC1* cDNA in a pBluescript KS vector) (19), except for MUT2 and SM2, for which the MUT mutant was used as the template. Construction of the MUT and TAG mutants was described previously (20). Mutagenic primers employed were 5′-GCTCCAAAGGCGATCCT- CAGTACACCGTGTC-3′ for EXT, 5′-ATAAAGGTGATTGAGAT- TATGAGGATC-3′ for TIT (removing the tiny uORF) and MUT2 (used on the MUT template, removing both uORFs), and 5′-GCGT- GAATAGAGTTATATCCGATCGAAGGTTG-3′ for TIN (removing the small uORF); the altered bases are underlined. A primer that disrupted the unique Apal site in pBluescript was used for the selection of mutants. The mutations were confirmed by DNA sequencing.

The MSS mutant was constructed using the MUT plasmid as template and the mutagenic primer 5′-CTCAAGAGGCAAATTCCTT- GAGTACACCG-3′ (inserted base underlined). The resulting cDNA has no small uORF, which has been replaced by a C-terminally extended tiny uORF, where the new tiny uORF stop codon coincides with the stop codon of the wild type small uORF. The FS mutant was constructed using a two-step mutagenesis. First, an extra nucleotide was inserted in the small uORF, near the 3′ end, using the mutagenic primer 5′-GCTC- CAAGAGGCAAATTCCTT-ATGAGGTGG-3′ (inserted nucleotide underlined). The newly created mutant plasmid was then used as the template for the second mutagenesis step, in which a nucleotide was deleted near the 5′ end of the small uORF sequence, with the mutagenic primer 5′-GAGATTATGAGTGATGCAAAATGGTTG-3′ (the deleted nucleotide, bracketed and in lowercase, was missing from the primer sequence). The FS plasmid has a frameshifted small uORF sequence such that 45 of the encoded 52 amino acids differ from the wild type, but the position of the small uORF within the cDNA is unaltered.

The F-S/TTG double mutant plasmid has the frameshifted small uORF and no tiny uORF and was produced using an overlapping PCR approach. Two first step PCR amplifications were carried out, using the FS plasmid as template and primer pairs 5′-TTAAGCCTTGTTCGCT- CCATCATAATCTCAATCACGCTTTTATATATAAATGAAGAAT- CAGTACCAGCGTGC-3′ and 5′-ATCGATCTTCATTTATATA- CAGTACCAGCGTGC-3′ for EXT. From the resultant product, a 0.4-kbp SalI-BamHI fragment was excised and used to replace the equivalent SalI-BamHI fragment consisting of the 5′ end of the small uORF sequence, with the mutagenic primer 5′-GCTCAAGAGGCAAATTCCTT-ATGAGGTGG-3′ for EXT, 5′-ATAAAGGTGATTGAGAT- TATGAGGATC-3′ for TIT (removing the tiny uORF) and MUT2 (used on the MUT template, removing both uORFs), and 5′-GCGT- GAATAGAGTTATATCCGATCGAAGGTTG-3′ for TIN (removing the small uORF); the altered bases are underlined. A primer that disrupted the unique Apal site in pBluescript was used for the selection of mutants. The mutations were confirmed by DNA sequencing.

**Plant Transformation and Growth Conditions**—For tobacco transformation, constructs in the pBin19 binary vector were introduced into *Agrobacterium tumefaciens* strain LBA4404 and used to inoculate leaf discs of *Nicotiana tabacum* cv. *xanthi* XHFD8 as previously described (23). Transgenic plantlets were selected on kanamycin and once rooted were transferred to soil in a greenhouse and grown at 25 °C with a 16-h light period. For *Arabidopsis* transformation the Columbia (Col-0) ecotype was grown in the greenhouse with long days (16 h of light) at 20 °C, and transformation was performed using the floral dip method (24). The different GUS constructs in pBI101 were introduced into the A. *tumefaciens* strain C58 GV3101. Transgenic seeds were selected by germination on kanamycin (50 μg/ml). Transforms were planted to soil and grown in a greenhouse, and analyses were performed on seeds from self-fertilization of these *T1* plants.

For tobacco seedling experiments, 100 seeds were surface-sterilized, washed, and transferred to a 1-liter conical flask containing 200 ml of Gamborg B5 medium (3.16 g/liter Gamborg B5 salts, 20 g/liter glucose, 0.5 g/liter MES, pH 5.7) and grown in the dark with shaking at 80 rpm and 25 °C for 3 weeks. These *T1* seedlings were a mix of the segregating homozygous, heterozygous, and azygous siblings derived from self-fertilization of the *T0* generation transgenic plants. For *Arabidopsis* seedling experiments, 500 *T2* sterilized segregating seeds from lines containing single transgene insertion loci, as determined by segregation analysis, were placed in a 1-liter flask of 200 ml of Gamborg B5 medium with half-strength sucrose (10 g/liter) and grown with shaking at 80 rpm.
and 25 °C for 10 days. All of the seedlings grown in the presence of polyniacines (and also controls) were washed five times with sterile water.

**Yeαst Transformation and Analysis**—The *S. cerevisiae* strain, 2602 (MATα ura3-52 his6 leu2) (obtained from H. Tabor, National Institutes of Health, Bethesda, MD), was used for GUS expression studies. Yeast cultures were grown aerobically at 25 °C in minimal SD medium. Yeast transformation was performed using a modified lithium acetate procedure (25). Transformed yeast strains were grown in 100 ml of SD medium lacking glucose, with 2% galactose, at 25 °C for 16 h. The cultures were divided into two 50-ml aliquots; the cells from one were pelleted and set aside at −70 °C for GUS activity assays. Total RNA was isolated from the remaining cells using the rapid RNA isolation method (26). For Northern analysis, 3 μg of total RNA was separated on 1.2% agarose formaldehyde denaturing gels and hybridized with the *gusA* sequence from pSLJ4D4 and rehybridized with the yeast TY element (27).

**In Vitro Transcription and Translation**—The wild-type *AdoMetDC1* cDNA and mutant cDNAs within the pBluescript KS vector were linearized at the 3′ end by digestion with NotI, before being *in vitro* transcribed using the RibonMax T3 kit (Promega). Reactions including 3 mM mG(5)ppp(5)GG RNA capping analogue (Invitrogen) were incubated at 37 °C for 4 h. Unincorporated NTPs and cap were removed from reactions by spin dialysis with Sephadex G-50, prior to quantification of RNA by spectrometry.

One microgram of each RNA transcript was translated in the wheat germ extract system (Promega), according to the manufacturer’s instructions. The reactions were performed in 40 μl, with 130 mM potassium acetate and 0.5 mM [35S]methionine (PerkinElmer Life Sciences). The reactions were incubated at 25 °C for 2 h and transferred to −20 °C. For visualization of *in vitro* translation products, aliquots of reactions were resolved on 10% SDS-polyacrylamide gels. Radiolabeled bands were quantified using a FujiBas 1500 phosphorimaging device.

**GUS Enzyme Assay**—Ground Arabidopsis tissue was assayed for GUS activity using the GUS-Light assay system (Tropix, Applied Biosystems, Warrington, UK). Tissue extracts were incubated with substrate for 1 h at room temperature, and light signal output was measured using a Lumat LB 9501 luminometer (Berthold, Pforzheim, Germany). Protein contents of extracts were measured using the method of Bradford (28), and GUS activity was expressed as relative light units/μg of protein. Tobacco tissue GUS activity was measured using a fluororescence assay (β-glucuronidase activity detection kit; Sigma-Aldrich) rather than a chelominescence assay because of discontinuance of the GUS-Light kit manufacture. Tissue extracts were incubated with the 4-methylumbelliferyl β-D-glucuronide substrate for 1 h at 37 °C. Fluorescence generated by the 4-methylumbelliferone product was measured in a Versafluo fluorometer (Bio-Rad). Duplicate assays were performed for each extract, two extractions were performed per sample, and the activity was expressed as nmol 4-methylumbelliferone produced h⁻¹ mg⁻¹ protein.

The cells from galactose-induced yeast cultures were resuspended in 60 mM NaHPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 7H₂O, pH 7.0, and vortexed on ice in the presence of acid-washed glass beads (425–600 μm). The cell debris was pelleted by centrifugation, and the extract was assayed using the GUS-Light system, as described above.

**RESULTS**

**The Plant *AdoMetDC* Small uORF Is Required for Polyamine-responsive Translational Regulation**—Previously we demonstrated that the plant *AdoMetDC* mRNA is post-transcriptionally regulated by excess polyniacines and that the small uORF participates in translational repression of the *AdoMetDC* mRNA under normal growth conditions (20). However, it was not determined whether the small uORF is involved in the post-transcriptional repression of *AdoMetDC* in response to polyniacines. To ascertain the role of the small uORF in polyniacine-responsive, post-transcriptional regulation of *AdoMetDC*, we used reporter constructs containing the *E. coli* β-glucuronidase (*gus*) reporter transcribed from the cauliflower mosaic virus 35 S promoter (Fig. 1). Inserted between the *gus* ORF and the promoter was either the Arabidopsis *AdoMetDC1* mRNA wild type 5′ leader sequence (SAM construct) or a site-directed mutant in which the small uORF AUGs and the tiny uORF stop codon were removed, thereby C-terminally extending the tiny uORF (MUT construct). The GUS construct lacks the 5′ leader sequence. For expression in yeast, the *AdoMetDC* 5′ leader-*gus* constructs are expressed from the GAL1 promoter (including the SAM-GUS and F-S/TTG constructs). For expression in the wheat germ *in vitro* system, the *AdoMetDC* 5′ leader constructs use the *AdoMetDC* proenzyme ORF as the reporter and the T3 promoter (includes the EXT construct). The promoter for each construct is indicated by the arrow shape. The tiny uORF, C-terminally extended tiny uORFs, and frameshifted small uORFs, all of which are in the same +1 reading frame relative to the original small uORF, are in light blue, and the wild type small uORF and C-terminally extended uORF of the EXT construct are in green. Frameshifted forms of the small uORF where the frameshift occurs only within the boundaries of the original small uORF are shown by the green-edged blue boxes (where the main part of the small uORF is in the same reading frame as the tiny uORF). The *gusA* ORF N-terminally fused in-frame to the small uORF in the SAM-GUS construct is indicated by the green and blue striped box. The reporter ORF of the EXT construct is found only as the *AdoMetDC* proenzyme version. See “Experimental Procedures” for full descriptions of site-directed mutations.

**FIGURE 1. Schematic representation of constructs used in transgenic plants, yeast, and the wheat germ *in vitro* system.** For expression in transgenic plants, the Arabidopsis *AdoMetDC* mRNA wild type 5′ leader and derived mutants are transcribed from the CaMV 35 S promoter with the *gusA* ORF downstream of the leader sequences for constructs SAM, MUT, TAG, FS, MSM, TIN, TTG, and MUT2. The GUS construct lacks the 5′ leader sequence. For expression in yeast, the *AdoMetDC* 5′ leader-*gus* constructs are expressed from the GAL1 promoter (including the SAM-GUS and F-S/TTG constructs). For expression in the wheat germ *in vitro* system, the *AdoMetDC* 5′ leader constructs use the *AdoMetDC* proenzyme ORF as the reporter and the T3 promoter (includes the EXT construct). The promoter for each construct is indicated by the arrow shape. The tiny uORF, C-terminally extended tiny uORFs, and frameshifted small uORFs, all of which are in the same +1 reading frame relative to the original small uORF, are in light blue, and the wild type small uORF and C-terminally extended uORF of the EXT construct are in green. Frameshifted forms of the small uORF where the frameshift occurs only within the boundaries of the original small uORF are shown by the green-edged blue boxes (where the main part of the small uORF is in the same reading frame as the tiny uORF). The *gusA* ORF N-terminally fused in-frame to the small uORF in the SAM-GUS construct is indicated by the green and blue striped box. The reporter ORF of the EXT construct is found only as the *AdoMetDC* proenzyme version.
than the MUT or GUS constructs. At 500 μM each of spd and spm there was a 43-fold translational repression of the SAM construct, a 5-fold repression of the MUT construct and 2-fold repression of the GUS construct.

Removal of the Tiny uORF Results in Translational Repression in the Absence of Added Polyamines—The 5′ tiny uORF overlaps the translationally repressive small uORF by one nucleotide, occluding the AUG of the small uORF, in a highly conserved configuration (Fig. 3). Arabidopsis AdoMetDC tiny uORF has gained a codon relative to rice, pine, and moss, resulting in two adjacent AUG codons for the tiny uORF (small uORF junction). The Arabidopsis tiny uORF termination codon overlaps the second AUG of the small uORF by one nucleotide. We removed the tiny uORF by changing its AUG to UUG using site-directed mutagenesis (TTG construct; Fig. 1). In young expanding leaves of T₀ generation transgenic tobacco plants, removal of the tiny uORF resulted in a 95% reduction in relative translation of the AdoMetDC 5′ leader in the absence of exogenously added polyamines (SAM versus TTG constructs; Fig. 4A). Elimination of the tiny uORF resulted in a 93% reduction in the relative translation of the AdoMetDC 5′ leader sequence in tobacco seedlings grown in liquid culture, in the absence of added polyamines (SAM versus TTG construct; Fig. 4B). The addition of polyamines at 500 μM each of spd and spm caused a 77% reduction in translational efficiency of the SAM construct in tobacco seedlings (Fig. 4B). This is a smaller repressive effect than observed in the experiment shown in Fig. 2, probably because of the addition of polyamines at day 13, rather than at day 0, for the experiment in Fig. 4B. A similar polyamine-independent translational repression (83%) due to elimination of the tiny uORF was observed with dark-grown Arabidopsis seedlings in liquid culture (SAM versus TTG construct; Fig. 4C), whereas the addition of 100 μM each of spd and spm caused a 72% reduction in translational efficiency of the wild type SAM construct (a lower concentration of polyamines was used with Arabidopsis because of greater growth sensitivity of seedlings to the added polyamines). These results demonstrate that the tiny uORF is a key component of the polyamine responsiveness of the AdoMetDC mRNA 5′ leader because its elimination causes translational repression of the downstream cistron in the absence of increased polyamine levels. With both tobacco and Arabidopsis seedlings, exogenously added polyamines further repressed the translational efficiency of the TTG construct where the tiny uORF was eliminated (a further 5.2% for tobacco and 14% for Arabidopsis; Fig. 4B and C).

The Small uORF Is a Sequence-dependent uORF—To unequivocally determine the importance of the small uORF-encoded peptide, two new constructs were made (Fig. 1) that frameshifted the small uORF but did not extend the reading frame beyond the normal stop codon. The small uORF of the FS construct was +1 frameshifted from its tenth nucleotide and reverted reading frame immediately before the small uORF stop codon, resulting in the alteration of 45 of the 52 encoded amino acids (see "Experimental Procedures"). The MSM construct was similar to FS except that the small uORF AUG was removed, and the extended tiny uORF terminated at the small uORF stop codon. In T₁ generation transgenic tobacco seedlings, the FS construct exhibited a small (1.8-fold) translational derepression attributable to frameshifting the small uORF. With both tobacco and Arabidopsis constructs were made (Fig. 1) that frameshifted the small uORF but did not extend the reading frame beyond the normal stop codon. The small uORF of the FS construct was +1 frameshifted from its tenth nucleotide and reverted reading frame immediately before the small uORF stop codon, resulting in the alteration of 45 of the 52 encoded amino acids (see "Experimental Procedures"). The MSM construct was similar to FS except that the small uORF AUG was removed, and the extended tiny uORF terminated at the small uORF stop codon. In T₁ generation transgenic tobacco seedlings, the FS construct exhibited a small (1.8-fold) translational derepression attributable to frameshifting the small uORF. The small uORF was significant (FS construct, p < 0.05; MSM construct, p < 0.01, single factor analysis of variance) in young tobacco leaves (FS and MSM versus SAM constructs; Fig. 4A), but removal of the tiny uORF (TTG versus SAM construct) caused a large increase in translational repression (Fig. 4). We reasoned that young expanding tobacco leaves need comparatively high levels of polyamine biosynthesis for growth and cell expansion, which might explain the relatively high translational efficiency of the wild type AdoMetDC mRNA leader in
these leaves. Additionally, tobacco uses polyamines for the biosynthesis of polyamine-hydroxycinnamic acid conjugates, probably used as defense molecules, and therefore may require a higher level of polyamine biosynthesis than Arabidopsis. To explore this idea, we made transgenic T2 Arabidopsis lines with the same constructs and measured relative translation in fully expanded rosette leaves harvested at the first appearance of a flower bud. The results presented in Fig. 5 confirm that in fully expanded Arabidopsis leaves the translational status of the AdoMetDC 5’ leader is the opposite of its status in young expanding tobacco leaves. Removal of the tiny uORF had no effect on translational efficiency (TTG versus wild type SAM construct). However, frameshifting the small uORF resulted in a 4-fold (MUT and FS) to 8-fold (MSM) translational derepression. This suggests that in expanded Arabidopsis leaves the AdoMetDC mRNA is translationally repressed under normal growth conditions, in contrast to young expanding tobacco leaves where it is translated efficiently. The 2-fold difference between the translational derepression seen with the FS versus MSM constructs may reflect the weaker initiation context of the tiny uORF AUG that initiates the new uORF in the MSM construct, leading to a larger proportion of scanning initiation complexes bypassing the MSM construct uORF and initiating at the AdoMetDC proenzyme ORF AUG.

To further test the hypothesis that the translational efficiency of the AdoMetDC 5’ leader was determined by the growth status of the plant tissue, Arabidopsis seedlings were grown in liquid culture for 2 weeks and then assessed for translational efficiency of the SAM, TTG, and FS constructs. In these rapidly growing Arabidopsis seedlings, removal of the tiny uORF (TTG construct) reduced relative translation by ~4.5-fold on average (SAM versus TTG, two independent transgenic lines for construct; Fig. 5B), whereas in fully expanded rosette leaves of the same transgenic lines, removal of the tiny uORF had no effect (Fig. 5C).
ing seedlings in the presence of 100 μM spd and spm reduced relative translation of the wild type SAM construct by 3.6-fold (Fig. 4C), indicating that this concentration of polyamines translationally represses the AdoMetDC mRNA leader, albeit not completely (compare with the TTG construct; Figs. 4C and 5B). In rapidly growing Arabidopsis seedlings the dynamic range of translational control appeared to be greater than that in the fully expanded rosette leaves.

The effect of the tiny uORF on relative translation of the AdoMetDC 5′ leader in the absence of the small uORF was examined in transgenic Arabidopsis rosette leaves. This was achieved by comparing a construct where both uORFs had been abolished by site-directed mutagenesis (MUT2 construct; Fig. 1) with a construct that contained only the tiny uORF (TIN construct; Fig. 1). The tiny uORF, in the absence of the small uORF, did not affect translational efficiency (Fig. 5A, compare TIN and MUT2 constructs). By comparing the FS construct with the MUT2 construct, it can be seen that the presence of both the tiny uORF together with the frameshifted small uORF also had little effect. This suggests that the translational repression of the wild type AdoMetDC 5′ leader is accomplished solely by the small uORF and its encoded peptide. The tiny uORF, which is not inhibitory per se, is required for modulating the degree of translational repression caused by the small uORF, possibly by influencing ribosomal recognition of the small uORF in response to polyamines.

The Small uORF Is Functional as a Translational Repressor in Yeast—The Arabidopsis AdoMetDC 5′ leader sequences fused to the GUS reporter shown in Fig. 1 were subcloned into the galactose-inducible yeast expression vector pYES2. After introduction into the S. cerevisiae strain 2602 and induction by galactose, the relative translation of each construct was determined. Replacing the small uORF with a C-terminally extended tiny uORF (MUT construct), introduction of a premature stop codon in the small uORF (TAG construct) (20), and removal of the small uORF (TIN construct) had no positive effect on relative translation (Fig. 6A). Elimination of both uORFs (MUT2 construct) increased relative translation ~2-fold. Removal of only the tiny uORF (TTG) caused a 20-fold reduction in relative translation, demonstrating that the Arabidopsis small uORF is functional in yeast but only when the tiny uORF is absent. To assess whether the small uORF is translated when the tiny uORF is present, the GUS reporter gene was fused downstream and in-frame with codon 14 of the small uORF (SAM-GUS construct; Fig. 1). Translational efficiency of the SAM-GUS construct was as low as the TTG construct, suggesting that the small uORF is not recognized in yeast when the occluding tiny uORF is present and that removal of the tiny uORF causes the small uORF to be recognized by the ribosome, resulting in translational repression. Fusion of the GUS reporter ORF to the N terminus of the small uORF eliminated GUS expression, indicating that very few ribosomes scanned past the tiny uORF AUG to reach the small uORF-GUS fusion ORF.

Because the small uORF does not seem to be translated in yeast in the presence of the tiny uORF, the sequence-dependent nature of small uORF function in yeast was assessed with a construct that was identical to the FS construct of Fig. 1 but with the tiny uORF eliminated (F-S/TTG construct; Fig. 1). The results presented in Fig. 6B show that the Arabidopsis AdoMetDC mRNA small uORF is also sequence-dependent in yeast. Comparison of the TTG and F-S/TTG constructs, where the only difference is the specific amino acid sequence encoded by the small uORF, shows that the wild type small uORF-encoded peptide is responsible for a 25-fold decrease in relative translation in yeast, similar to its effect in tobacco leaves (Fig. 4). The wild type small uORF has one relatively rare codon for yeast at the 23rd position (CUC), which has a codon adaptation index (CAI) value of 21% (30). However, the frameshifted small uORF in the F-S/TTG construct contains two relatively rare codons: the 4th codon (CGA, CAI value 15%) and the 21st codon (CUC, CAI value 21%). It is reasonable to assume that the translational derepression attributable to frameshifting the small uORF is not due to elimination of rare codons. When the yeast lines were grown in the presence of 500 μM spd and spm, no polyamine-dependent translational repression of the wild type SAM construct was observed, and no effect on the endogenous AdoMetDC activity was detected, indicating that the yeast AdoMetDC is insensitive to polyamines (results not shown).

The Small uORF Is Translated in Vitro—To establish directly whether the AdoMetDC small uORF is translated, a wheat germ in vitro translation system was programmed with the entire wild type AdoMetDC1 mRNA or site-directed mutant mRNAs with modified 5′ leaders depicted in Fig. 1. These mutant cDNA plasmids contain the AdoMetDC proenzyme ORF. Translation reactions were performed in the presence of [35S]methionine, and products were visualized by SDS-PAGE followed by autoradiography. The results presented in Fig. 7 show that a peptide of the size predicted for the small uORF (~5.5 kDa) is produced from mRNAs in which the small uORF is present (SAM and TTG cDNAs). When the small uORF is truncated (TAG), no band was detectable, but when the reading frame of the small uORF is maintained and the original stop codon mutated so that the small ORF extends to a

![FIGURE 6. Relative translation of the Arabidopsis AdoMetDC1 5′ leader constructs in yeast. A, reporter constructs were transcribed from the GAL1 promoter and induced with galactose. The relative translation was calculated as GUS activity divided by gusA mRNA level for each construct. gusA mRNA levels were normalized to Ty mRNA levels for each construct. The data represent the mean values of three independent transformants, and the mean value of the SAM construct was set at 1.0. Three micrograms of total RNA/lane was used for RNA gel blots from which the relative mRNA levels were quantified. B, importance of the small uORF-encoded amino acid sequence for translational repression in yeast. The F-S/TTG construct lacks the tiny uORF and the small uORF is frameshifted so that 45 of 52 amino acids are altered. Three micrograms of RNA/lane was used for the RNA gel blots for mRNA quantification. The mean value for the GUS construct was set at 1.0.](image-url)
DISCUSSION

The small uORF predicted amino acid sequence, conserved between Arabidopsis, rice, Pinus taeda, and moss, representing more than 400 million years of divergence (31), strongly suggested that the small uORF may represent a sequence-dependent uORF, where the encoded peptide is essential for function. To test the sequence-dependent nature of the small uORF, the FS and MSM constructs were created, where the small uORF terminated at the original small uORF stop codon and was frame-shifted from two codons downstream of the small uORF AUG (FS) or C-terminally extended from the tiny uORF AUG (MSM). It was important to assess the sequence dependence of the small uORF with constructs maintaining the original stop codon rather than with constructs that prematurely truncated or C-terminally extended the small uORF, because the sequence immediately downstream of the stop codon of sequence-independent uORFs is known to affect reinitiation competence of post-termination ribosomes (32). By comparison of these constructs with the wild type and TTG constructs, where the occluding tiny uORF had been eliminated, we demonstrated that the Arabidopsis small uORF is a sequence-dependent uORF, i.e. the sequence of the encoded peptide is essential and is the first such uORF to be identified experimentally in plants. We were also able to demonstrate that the small uORF was functional as a translationally repressive sequence-dependent uORF in yeast, suggesting that the target of the small uORF peptide in the translation machinery has a highly conserved function, such as the peptidyl transferase center of the ribosome.

The proposal that the inhibitory effect of the plant AdoMetDC small uORF is due solely to its unusually long size rather than its amino acid sequence (33) is no longer tenable. However, the length of the small uORF peptide does suggest that, like the E. coli SecM peptide (34), it could interact with the ribosome exit tunnel. The C. reinhardtii small uORF predicted peptide has some features conserved with the terrestrial plant small uORF peptides, such as the C-terminal proline and serine immediately before the termination codon, other conserved proline positions, and the size of the small uORF peptide. Arabidopsis and C. reinhardtii last had a common ancestor possibly as long ago as 1.1 billion years (31).

In transgenic plants and yeast, when the tiny uORF is eliminated, the small uORF constitutively represses translation of the downstream cistron. This indicates that the translationally repressive function of the small uORF is independent of excess polyamine levels and that the tiny uORF is essential for polyamine response and engagement of the small uORF-mediated translational repression. One possible explanation for our data, consistent with the observed results, is that elevated polyamine levels cause increased ribosomal recognition of the small uORF, resulting in translational repression of the downstream cistron. Two conserved features of the tiny uORF/small uORF configuration are that the
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small uORF is in the +1 reading frame relative to the tiny uORF, and the tiny uORF has a weaker AUG sequence context for translation initiation than the small uORF AUG. For translation of the small uORF to occur, two possible mechanisms are conceivable: programmed −1 ribosomal frameshifting from the tiny to the small uORF, or, ribosomal leaky scanning past the weak tiny uORF AUG to that of the stronger small uORF AUG. A well known example of polyamine-stimulated programmed translational frameshifting is the ornithine decarboxylase antizyme (35). The antizyme mRNA level is unaffected by changes in polyamine levels, but a polyamine-stimulated translational frameshifting results in production of the full-length antizyme protein that then binds to the ornithine decarboxylase monomer and targets it for degradation by the 26 S proteasome (36). In both mammalian and Schizosaccharomyces pombe antizyme mRNA, the polyamine-stimulated frameshifting is +1 (37). However, there are several features of the plant AdoMetDC tiny uORF/small uORF cassette that make frameshifting seem an unlikely explanation. First, the tiny uORF AUG context is weak, and any leaky scanning would undermine a frameshifting mechanism. Second, the frameshift for most plant AdoMetDCs would have to take place on the codon immediately after the tiny uORF initiator AUG. There is little nucleotide sequence conservation either side of the tiny uORF AUG to suggest a conserved “shifty” sequence. Third, the frameshift would have to be −1, in contrast to the +1 of antizyme; the C. reinhardtii tiny/small uORF frameshift would have to be +1.

The alternative explanation for the ribosomal switch from the tiny uORF to the small uORF is ribosomal leaky scanning. Excess polyamines cause a decrease in the translational efficiency of control constructs (see GUS construct in Fig. 2), which could be explained by a general effect on ribosomal recognition of AUGs. If excess polyamines caused a general increase in leaky scanning, this would be damaging for the cell. The weak AUG/strong AUG configuration of the AdoMetDC mRNA uORFs could act as a hair trigger for detecting and responding to polyamine-stimulated general leaky scanning. It is interesting to note that polyamines have been shown to inhibit phosphorylation of eIF2α (38), a translation initiation factor subunit known to be involved in the recognition of AUGs (39). Alternatively, a polyamine-stimulated leaky scanning mechanism could be specific to the AdoMetDC mRNA. Polyamines might bind to a specific sequence on the mRNA in a manner analogous to the binding of metabolites such as AdoMet, vitamins, purines, and amino acids to ribosensor sequences in bacterial mRNAs (40–42). Ribosensor motifs can alter the ribosomal accessibility of the mRNA Shine Dalgarno sequence upon binding of metabolites. By analogy, binding of polyamines to the AdoMetDC mRNA could change the mRNA secondary structure, allowing differential recognition of the tiny and small uORF AUGs. In support of a mechanism based on leaky scanning, the tiny uORF/small uORF cassette is not responsive to polyamines in yeast, where AUG selection is more stringent (33).

When the tiny uORF was removed (TTG construct), exogenous polyamines still caused a further, small decrease in translational efficiency in tobacco and Arabidopsis seedlings. This suggests that changes in ribosomal initiation complexes bypass the strong AUG of the small uORF because of polyamine-mediated increased leaky scanning. These same scanning initiation complexes may also bypass the gusA ORF AUG, resulting in a decrease in GUS activity that would be perceived as a decrease in translational efficiency. That excess polyamines cause a decrease in translational efficiency of the TTG construct, containing only the small uORF, argues strongly for ribosomal leaky scanning rather than ribosomal frameshifting as being the sensor of polyamine levels.

We propose the following model for the function of the tiny and small uORF module. In low polyamine conditions the scanning preinitiation complex recognizes the tiny uORF AUG, translates the tiny uORF, and terminates and then efficiently reinitiates at the downstream AUG of the AdoMetDC proenzyme ORF, thereby synthesizing AdoMetDC enzyme and raising the level of polyamines. When polyamine levels are too high, the scanning preinitiation complex either scans past the weak AUG of the tiny uORF and then recognizes the stronger small uORF AUG, or the ribosome recognizes the tiny uORF AUG but then immediately undergoes −1 frameshifting to translate the inhibitory small uORF, resulting in translational repression of the downstream proenzyme ORF and decreased AdoMetDC and polyamine synthesis. In this model, the tiny uORF provides rapid amplitude modulation for the polyamine response mechanism by allowing bypass of the constitutively inhibitory sequence-dependent small uORF when polyamine levels are low. Inherent in this model is a stochastic element that produces gradual changes in AdoMetDC mRNA translation by altering the proportion of the AdoMetDC mRNA population being translated or repressed. It is likely that the general translational machinery is the cellular polyamine sensor and that the tiny uORF/small uORF module amplifies and transduces the free polyamine concentration signal that regulates AdoMetDC translation and polyamine biosynthesis. In contrast to the model of AdoMetDC translational regulation presented here, Hu et al. (43) have suggested recently that the tiny and small uORFs show the same function in response to polyamines. We note that the level of exogenously added spermine used in their study was 20–100-fold higher than the physiological levels employed in our study.

Acknowledgments—We thank Julie Hofer and Robert Sablowski for helpful comments on the manuscript and the Kazusa DNA Research Institute for the C. reinhardtii expressed sequence tag.

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