Abrogation of Upstream Open Reading Frame-mediated Translational Control of a Plant S-Adenosylmethionine Decarboxylase Results in Polyamine Disruption and Growth Perturbations*

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S-Adenosylmethionine decarboxylase (AdoMetDC) is a key enzyme in polyamine biosynthesis. We show that the plant AdoMetDC activity is subject to post-transcriptional control by polyamines. A highly conserved small upstream open reading frame (uORF) in the AdoMetDC mRNA 5′ leader is responsible for translational repression of a downstream β-glucuronidase reporter cistron in transgenic tobacco plants. Elimination of the small uORF from an AdoMetDC cDNA led to increased relative translational efficiency of the AdoMetDC proenzyme in transgenic plants. The resulting increased activity of AdoMetDC caused disruption to polyamine levels with depletion of putrescine, reduction of spermine levels, and a more than 400-fold increase in the level of decarboxylated S-adenosylmethionine. These changes were associated with severe growth and developmental defects. The high level of decarboxylated S-adenosylmethionine was not associated with any change in 5′-methylcytosine content in genomic DNA and S-adenosylmethionine levels were more or less normal, indicating a highly efficient system for maintenance of S-adenosylmethionine levels in plants. This work demonstrates that uORF-mediated translational control of AdoMetDC is essential for polyamine homeostasis and for normal growth and development.

S-Adenosylmethionine decarboxylase (AdoMetDC1; EC 4.1.1.50) is a key enzyme in the biosynthesis of the polyamines spermidine and spermine. Polyamines are multivulcent cations implicated in a wide range of cellular physiological processes including chromatin organization, mRNA translation, cell proliferation, and apoptosis. Most plants form putrescine (1,4-diaminobutane) indirectly from arginine and directly from ornithine. Spermidine is formed from putrescine and spermine from spermidine by successive addition of anpropyl groups derived from decarboxylated S-adenosylmethionine (AdoMet).

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‡ The abbreviations used are: AdoMetDC, S-adenosylmethionine decarboxylase; uORF, upstream open reading frame; MES, 4-morpholineethanesulfonic acid; dansyl, 5-dimethylaminonapthalene-1-sulfon-nyl; NL, no leader; HPLC, high performance liquid chromatography; GUS, β-glucuronidase; CaMV, cauliflower mosaic virus.

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responsibility for the transcriptional repression of a downstream cistron in transgenic plants; (iii) abrogation of small uORF-mediated translational regulation in transgenic plants causes an increased translation of the downstream AdoMetDC ORF, resulting in increased enzyme activity and decarboxylated AdoMet levels, polyamine disruption, and severe growth perturbations.

EXPERIMENTAL PROCEDURES

Plant Material and Growth Conditions—For Arabidopsis studies, the Columbia ecotype (Col-0) was grown in a greenhouse with a 16-h light period. The Arabidopsis cell suspension culture (obtained from J. Medford, University of Cambridge, United Kingdom) was grown in a 1-liter flask in liquid medium containing MS salts (19), 1% sucrose, 0.5 mg/liter naphthalene acetic acid, 0.05 mg/liter kinetin, pH 5.8, at 25 °C in the dark, shaking at 80 rpm. For in vitro growth of tobacco, seeds of Nicotiana tabacum cv. Xanthi HFXD8 were surface sterilized and germinated on medium containing MS salts, 2% sucrose, 0.5 g/liter MES, 8 g/liter Bacto-agar, pH 5.7, and grown at 25 °C with 16 h daylength. For leaf disc experiments, young leaves about 8 cm in length were washed in 10% bleach with a few drops of detergent for 15 min and then rinsed three times in sterile water. Leaf discs of 8 mm diameter were cut from the leaves and placed on solid MS medium without hormones in Petri dishes.

Site-directed Mutagenesis—The various site-directed mutants of the AdoMetDC1 cDNA were produced using the Chameleon double-stranded mutagenesis kit (Stratagene), following the manufacturer's instructions. Mutants were constructed using the SAMDC1 plasmid (which contains the wild type AdoMetDC1 cDNA in a pBluescript KS plasmid). Mutagenic primers employed were 5'-CGCGTAATGACAATATTGACAAGCTCCCTC-3' for the MUT construct and 5'-CGAAGCTCTCCCTCCTGGTTAGCATTTGGACGGC-3' for the TAG construct. A primer which disrupted the unique ApaI site in pBluescript was used for selection of mutants. Mutations were confirmed by DNA sequencing.

Plasmid Construction—The 5' leader sequences from SAMDC1 and the site-directed mutants were PCR amplified using the primers 5'-TTAGAGCTCTCACTAATGTTGCTCT-3' (SacI site underlined) and 5'-CTCCATTGCTCCTGCTTGTGTTGTGAGCCG-3' (NcoI site underlined). PCR products were checked for errors by sequencing. SacI-NcoI fragments containing the 5' leader were then used to replace the tobacco mosaic virus II sequence in the pCI118-based vector, pSLJ4D4 (21). From the resultant plasmids, 4.4-kilobase pair EcoRI-HindIII fragments containing the CaMV 35S RNA promoter, the 5' leader variants, the Escherichia coli β-glucuronidase (GUS) coding sequence, and the octopine synthase terminator were cloned into the Agrobacterium tumefaciens binary vector, pBin19. The GUS sequence was subsequently amplified from pSLJ4D4 using the primers 5'-CGAAGCTCTCCCTCCTGGTTAGCATTTGGACGGC-3' for the MUT construct and 5'-CGAAGCTCTCCCTCCTGGTTAGCATTTGGACGGC-3' for the TAG construct. A primer which disrupted the unique ApaI site in pBluescript was used for selection of mutants. Mutations were confirmed by DNA sequencing.

RNA Isolation and RNA Gel Blot Analysis—Plant tissue was ground to a fine powder in liquid nitrogen, an aliquot was set aside at −70 °C for GUS or AdoMetDC activity assays, and the remainder was used to prepare total RNA as described previously (22). For RNA gel blot analysis, 10 μg of total RNA was size-fractionated on 1.2% agarose-formaldehyde denaturing gels, and blotted onto Hybond-N membranes (Amersham Biosciences). Blots were probed with the 1.9-kilobase pair NeoI-XhoI fragment of the GUS sequence from pSLJ4D4 (21), the 1.8-kilobase pair SacI-XhoI fragment containing the AdoMetDC1 cDNA, or the 0.7-kilobase pair NeoI fragment containing the PCR amplified cDNA fragment with a ubiquitin sequon as the hybridization probe, as described previously (22).

GUS Enzyme Assay—Ground plant tissue was assayed for GUS activity using the GUS-Light assay system (Tropix, Applied Biosystems, Warrington, UK), following the manufacturer's instructions. Tissue extracts were incubated with substrate for 1 h at room temperature, and light signal output was measured using a Lumat LB9501 luminometer (Berthold, Pforzheim, Germany). Protein contents of extracts were measured using the method of Bradford (23), and GUS activity was expressed as relative light units per μg of protein.

AdoMetDC Enzyme Assay—Ground plant tissue was assayed for AdoMetDC activity as described previously (22). Assays were performed at 37 °C for 45 min, and AdoMetDC activity was determined by measurement of 14CO2 release from S-adenosyl-L-[14C]methionine (Amer sham Biosciences). Protein contents of extracts were measured using the method of Bradford (23), and enzyme activities were expressed as nanomoles of CO2/h/mg of protein.

Measurement of Polyamines—Polyamines were extracted once from fresh leaf material (10 ml) in chloroformic acid containing 1× 10−4 M 1,7-diaminoheptane as an internal standard as described previously (20). Polyamines were dianlysed overnight and sample aliquots of 200 μl were incubated with 100 μl of saturated Na2CO3, and 600 μl of dianyl chloride (10 mg/ml in aceton) for 16 h in the dark in open tubes to allow gradual evaporation of the aceton. Excess dianyl chloride was removed by 30 min incubation with 150 μl of proline (300 mg/ml). The reaction was then extracted with 1 ml of toluene, centrifuged for 5 min at 13,000 × g, and 800 μl of the upper phase was dried with nitrogen and resuspended in 500 μl of acetonitrile. Samples were filtered through Acrodisc CR PTFE filters (Gelman Sciences, Northampton, UK). Dianlysed polyamines were separated by HPLC using a Phenospheresphere C18 ODS (2) column (250 × 4.6 mm; Phenomenex, Macclesfield, Cheshire, UK) with fluorescence detection (excitation wavelength 340 nm, emission wavelength 510 nm). Solvent A was HPLC-grade water, solvent B was acetonitrile, and the gradient was run for 50 min at a flow rate of 1.2 ml/min with the following concentrations: t = 0 min, 40% A, 60% B; t = 25 min, 0% A, 100% B; t = 40 min, 40% A, 60% B; t = 50 min, 40% A, 60% B.

Measurement of Global DNA 5'-Methylcytosine Content—DNA samples (20 μg in 50 μl water) were hydrolyzed for 14 h at 37 °C with 42 μl of P1 nuclease (Sigma) that was in 55 μl of 30 mM sodium acetate buffer, pH 5.3, and 20 μl of 10 mM ZnCl2 to form a total volume of 125 μl. The 5'-phosphate of the free nucleotides was removed by hydrolysis with bacterial alkaline phosphatase (Sigma) for 2 h at 37 °C in a total volume of 100 μl of 300 mM Tris-OH buffer, pH 8.7, containing 3.5 units of enzyme. Samples were then filtered through a 0.45-μm PTFE membrane (Gelman Sciences). The filtered samples were loaded onto a Supelcosil LC 18-S (5 μm × 4.6 mm) reverse phase HPLC column (Phenomenex, Macclesfield, Cheshire, UK). Nucleosides were separated on an isocratic gradient and quantified by UV detection: buffer A was 0.05 M KH2PO4, pH 4.0, 8% MeOH and buffer B was 70% methanol. Column temperature was 25 °C and UV acquisition was at 254 and 280 nm. 5'-Methyl-2'-deoxycytidine and 2'-deoxycytidine standards were obtained from Sigma.

Measurement of AdoMet and Decarboxylated AdoMet Content—AdoMet and decarboxylated AdoMet were measured by reverse phase HPLC. For AdoMet measurement it was essential to keep sample extracts frozen until immediately before injection onto the column because of the liability of AdoMet. Samples of frozen leaf powder were extracted with 1 ml of 5% trichloroacetic acid per 400 mg of powder. Extracted samples were centrifuged at 10,000 × g for 15 min to clear cell debris. The supernatant was then centrifuged at 13,000 × g to further remove debris and the supernatant was filtered. Decarboxylated AdoMet was a kind gift of Dr. B. Blessington, University of Bradford, UK, and Prof. A. E. Pegg, Hershey Medical School, University of Pennsylvania, and AdoMet was obtained from Sigma. AdoMet and decarboxylated AdoMet were identified by UV detection in the presence of a Luna 5 μm ODS (2) 150 × 4.6-mm reverse phase HPLC column (Phenomenex). The solvent gradient was formed from buffer A (0.1 M sodium acetate, pH 4.5, 10 mM 1-octanesulfonic acid) and buffer B (0.2 M sodium acetate, pH 4.5, acetonitrile (10:3) with 10 mM octanesulfonic acid). The gradient was formed as follows: t = 0, 100% A, 0% B; t = 30, 0% A, 100% B; t = 40, 0% A, 100% B; t = 45, 100% A, 0% B with a flow rate of 1.5 ml/min. UV acquisition was at 259 nm.

RESULTS

The Plant AdoMetDC Is Post-transcriptionally Regulated by Polyamines—To determine the relevance of the conserved overlapping uORFs in the plant AdoMetDC mRNA 5' leader to translational control, we looked for evidence of post-transcriptional regulation. AdoMetDC is initially synthesized as an inactive proenzyme and is autokatallytically processed to produce the mature form of the enzyme containing a covalently linked
pyruvoly cofactor at the N terminus of the α-subunit. The processing reaction of the potato and Arabidopsis proenzymes is very rapid and, unlike the mammalian enzyme, is not regulated by the polyamine precursor putrescine (24). The plant AdoMetDC activity is therefore likely to be a reliable indication of the amount of AdoMetDC protein. AdoMetDC1 is the more actively expressed of two expressed AdoMetDC genes in Arabidopsis (6). Fig. 1A shows the variation in AdoMetDC activity detected in different organs of Arabidopsis. The ratio of AdoMetDC activity to ubiquitin-normalized AdoMetDC1 mRNA levels in leaves, stems, roots, and flowers is 1, 0.8, 0.7, and 1.2, respectively, indicating that spatial activity of AdoMetDC1 is largely correlated with steady-state mRNA levels.

It is known that application of polyamines to tobacco suspension culture cells results in decreased AdoMetDC activity (25). We examined AdoMetDC activity and steady-state mRNA levels in stationary phase 10-day-old Arabidopsis suspension culture cells simultaneously treated with 0.5 mM spermidine and spermine (Spd + Spm) or water (Control) for 16 h. C, tobacco plantlets grown in vitro in the presence of polyamines. Seeds were germinated on normal MS growth medium (Control) or medium supplemented with 0.5 mM each of spermidine and spermine (Spd + Spm), and all above ground parts of the plants were harvested after 41 days. RNA gel blots were performed using 10 μg of total RNA per lane (A, AdoMetDC; U, ubiquitin). Hybridization signal intensities were quantified using a FujiBas 1500 PhosphorImager and AdoMetDC mRNA values were normalized to ubiquitin with data presented as photostimulated luminescence (PSL).

The SAM construct contained the wild type AdoMetDC1 5′ leader. The MUT construct contained a leader sequence in which the small uORF was abolished and replaced by the tiny uORF, which was extended downstream to 66 codons (in the +1 reading frame relative to the small uORF and extending 31 nucleotides downstream of the small uORF stop codon). The TAG construct contained a leader sequence with the small uORF C-terminal truncated to 25 codons by the introduction of a UAG nonsense codon (see Fig. 2, B and C, for site-directed mutations).

As shown by the results of the MUT construct depicted in Fig. 3, elimination of the small uORF caused a 3-fold derepression of GUS translational efficiency in leaves of transgenic plants. The 5-fold translational depression seen with the TAG construct indicates that the C-terminal half of the small uORF peptide or the sequence immediately 3′ of the termination codon is essential for translation inhibition. Together these results suggest that the plant AdoMetDC mRNA is translationally repressed in planta and that the small uORF is responsible for translational repression of the downstream cistron.

**Deregulated Translation of AdoMetDC in Transgenic Plants**

**Results in Increased Enzyme Activity and Severe Growth Abnormalities**—To investigate the biological significance of the translational control of AdoMetDC expression observed in this study, we produced transgenic tobacco plants expressing either the wild type Arabidopsis AdoMetDC1 cDNA, or AdoMetDC1 cDNA with the 5′ leader truncated from 505 to 58 nucleotides (NL), or with the small uORF C-terminal truncated from 53 to 25 codons by introduction of a premature nonsense codon (TAG). The cDNAs were cloned downstream of a CaMV 35S RNA promoter to allow constitutive expression in transgenic tobacco plants. Transgenic T0 tobacco plants were allowed to flower and self-fertilize. Progeny segregated in a mendelian
manner into transgenic progeny contained the transgene and syngenic progeny without the transgene.

Transgenic plants from two independent lines for each of the SAM, TAG, and NL cDNAs were analyzed for AdoMetDC activity. Mean AdoMetDC activity recorded in the segregating syngenic siblings was subtracted from the activity in the transgenic siblings to give a measure of activity because of the transgene. Table I shows the relative transgene mRNA, AdoMetDC activity, and relative AdoMetDC translational efficiency values for representative individuals from each of the transgenic lines. Each of the modified AdoMetDC1 cDNA lines shows an increase of relative translational efficiency of between 5- and 18-fold above that of wild type AdoMetDC1 cDNA overexpressing lines 556 and 557. The higher translational efficiency of the two NL lines compared with the TAG lines is likely because of the inhibitory influence of the long AdoMetDC1 35' leader sequence. Secondary structure in the maize uORF-containing Lc mRNA leader sequence is responsible for half of the translational repression conferred by the leader sequence (26). Once translational regulation is removed, AdoMetDC activity is dependent on mRNA levels, which are subject to position effects.

Plants overexpressing the wild type SAM construct (lines 556 and 557) exhibited a normal morphological phenotype. In contrast, both of the NL lines (754 and 756), and one of the TAG lines (850) exhibited severely abnormal phenotypes, which segregated into two levels of severity for each line and which were clearly visible as growth differences in seedlings (Fig. 4, A–C). The NL756 plants displayed the most extreme morphological phenotype and the NL754 plants the mildest. The phenotype displayed by the NL756 line was usually lethal with the presumed homozygous plants dying before they attained 2 cm and the putative heterozygous plants dying usually before they reached 5 cm. Segregating normal syngenic plants of line NL756 flowered at a height of ~100 cm. All transgenic plants were stunted with reduced internode length (Fig. 4, D–F) and with wrinkled and curled leaves in the NL754 and NL756 lines. The abnormal morphological and growth phenotype was more marked in the second generation T1 plants that had passed through meiosis than in the first generation T0 plants that had been regenerated from tissue culture. Furthermore, transgenic plants of the T2 generation (resulting from self-fertilization of T1 plants) were yet more severe in their growth defects. Segregating T1 progeny of the TAG850 line shown in Fig. 4 can be compared with the corresponding T2 progeny shown in Fig. 4G. Transgenic T1 plants were sensitive to higher temperatures of the greenhouse in summer. By moving the plants to an air-

![Fig. 2. Structure of the *Arabidopsis* AdoMetDC1 mRNA. A, schematic representation of AdoMetDC1 mRNA. The tiny uORF consists of four codons, and is represented by a black box; the small uORF consists of 53 codons, and is represented by a white box. B, sequences flanking and containing the two uORFs from the wild type cDNA (SAM) and the site-directed mutants used in this study. Numbers refer to nucleotides, numbered from the 5' end of the mRNA. ATG initiation codons are shown in uppercase letters; termination codons are underlined. Mutant sequences identical to wild type AdoMet sequence are shown as dashes. C, schematic representation of the site-directed mutants shown in B. The white arrow represents the CaMV 35S RNA promoter, the hatched block represents a downstream ORF, the black box the tiny uORF and derivatives, and the white box the small uORF and derivatives.](http://www.jbc.org/)
conditioned greenhouse maintained at 21 °C, more plants survived and four NL756 plants flowered (out of more than 100 transgenic plants). Besides greatly delayed flowering, flower morphology was uniformly abnormal in all lines, consisting of darker, more intense pigmentation and smaller petals that seemed to be unable to expand completely so that the corolla did not open fully (Fig. 4H). Flowers were less fertile and the inside and outside of the corolla tube and the filaments of the stamens were pigmented. The inhibition of expansion also occurred in transgenic leaf discs placed on MS solid medium without hormones in vitro. NL754 transgenic leaf discs were inhibited in cell expansion after 6 weeks when compared with leaf discs of syngenic sibling plants (Fig. 4I). When transgenic scions (shoots) of NL754, NL756, and TAG850 T1 plants were grafted onto normal root stocks, no attenuation of the transgene. The value for 556-M was set as 1.00.

\[ \text{AdoMetDC translational efficiency in leaves of T1 transgenic tobacco plants} \]

| Construct | Plant | Relative AdoMetDC mRNA | Relative AdoMetDC activity | Relative AdoMetDC translational efficiency |
|-----------|-------|------------------------|---------------------------|------------------------------------------|
| SAMDC1    | 556-M | 1.00                   | 1.00                      | 1.00                                     |
| SAMDC1    | 557-Q | 0.55                   | 0.72                      | 1.31                                     |
| SAMDC1NL  | 754-M | 0.94                   | 16.84                     | 17.06                                    |
| SAMDC1NL  | 754-T | 0.60                   | 11.22                     | 18.70                                    |
| SAMDC1NL  | 756-P | 3.42                   | 48.52                     | 14.19                                    |
| SAMDC1NL  | 756-R | 2.79                   | 18.93                     | 6.78                                     |
| SAMDC1TAG | 850-C | 1.09                   | 9.54                      | 8.75                                     |
| SAMDC1TAG | 852-M | 0.24                   | 1.37                      | 5.71                                     |

\^ Individuals designated with the same number are from the same transgenic line.
\^ AdoMetDC1 mRNA levels were normalized to ubiquitin for each plant. The value for 556-M was set as 1.00.
\^ AdoMetDC activity was measured as nanomole of CO2 released per mg of total protein. For each plant, the mean AdoMetDC activity recorded in segregating syngenic siblings was subtracted from the total AdoMetDC activity level to give a measure of activity due to the AdoMetDC1 transgene. The value for 556-M was set as 1.00.
\^ Relative translational efficiency is the relative AdoMetDC activity divided by ubiquitin-normalized AdoMetDC1 mRNA. The value for 556-M was set as 1.00.

AdoMetDC is involved solely in polyamine biosynthesis but the substrate AdoMet is the main methyl donor in transmethylation reactions and in plants it also serves as a substrate for ethylene biosynthesis and allosteric activation of threonine synthase (28–30). It is therefore not surprising that the plant AdoMetDC might be subject to multiple levels of regulation.

The increasing phenotypic abnormalities seen in advancing generations and the nature of the morphological abnormalities were reminiscent of the Arabidopsis DNA methylation mutant adm1 (27). One of the main functions of AdoMet is to supply the methyl group for transmethylation reactions and overexpression of AdoMetDC has the potential to deplete AdoMet to an extent that might affect the level of genomic DNA methylation. We used the same plant material employed for the polyamine analysis in Table II to investigate the level of 5’-methylcytosine in the T1 segregating progeny. As detected by reverse phase HPLC, the content of 5’-methylcytosine relative to unmethylated cytosine was the same in transgenic and syngenic plants (Table III).

For further biochemical analyses we worked with the T2 generation (obtained from self-fertilization of T1 parent plants). Absolute levels of AdoMetDC activity were still elevated in the transgenic plants, by 7-fold in NL756 and 2.5-fold in TAG850 plants (Table IV). Polyamine disruption was more pronounced in the T2 generation and putrescine was barely detectable (Table V). In the TAG850 transgenic plants, the spermine to spermidine ratio was reduced 3-fold but spermidine levels were relatively similar between syngenic and transgenic siblings.

AdoMet and decarboxylated AdoMet were analyzed by reverse phase HPLC in the same T2 plant material described above. Surprisingly the elevated AdoMetDC activity in the NL756 transgenic plants did not result in changes to the AdoMet content (Fig. 5A) although the TAG850 plants showed more than 2-fold decrease in AdoMet concentration (Fig. 5A). In stark contrast, the content of decarboxylated AdoMet was massively increased in both lines (Fig. 5B). In syngenic plants of the TAG850 line decarboxylated AdoMet was not detectable but two of the NL756 syngenic pooled samples contained detectable decarboxylated AdoMet at levels of 0.0018 and 0.03 nmol/g fresh weight. In the NL756 syngenic plants the mean decarboxylated AdoMet level represented less than 2.5% of the AdoMet pool but in the NL756 transgenic plants decarboxylated AdoMet exceeded AdoMet by 10-fold and by 2.8-fold in the TAG850 plants.

**DISCUSSION**

AdoMetDC is involved solely in polyamine biosynthesis but the substrate AdoMet is the main methyl donor in transmethylation reactions and in plants it also serves as a substrate for ethylene biosynthesis and allosteric activation of threonine synthase (28–30). It is therefore not surprising that the plant AdoMetDC might be subject to multiple levels of regulation. Two lines of evidence are presented here suggesting that the highly conserved small uORF is responsible for the translational regulation of the plant AdoMetDC. First, in leaves of stably transformed tobacco plants, elimination of the small uORF or removal of the C-terminal 28 amino acids results in translational derepression of a downstream GUS reporter ORF. It is not the presence of an uORF per se that is required for translational repression. For instance, the MUT construct replaces the small uORF with a C-terminal extended tiny uORF in a different reading frame but it is not translationally repressive. Furthermore, the small uORF amino acid sequence is highly conserved (6), with most nucleotide changes between species occurring in the third “wobble” position of codons. However, we cannot say with the present constructs that the small uORF functions in a sequence-dependent manner. The MUT construct replaces the small uORF with a +1 reading frame, C-terminal extended tiny uORF terminating 31 nucleotides.
Fig. 4. Overexpression of Arabidopsis AdoMetDC1 in transgenic tobacco. A–C, T1 segregating progeny of self-fertilized T0 plants: A, NL756; B, TAG850; and C, NL754. D–F, T1 segregating progeny of self-fertilized T0 plants at a stage when the syngenic plants have flowered (syngenic plants to the left, transgenic plants to the right showing variable penetration of the aberrant growth phenotype): D, NL756, E, TAG850, and F, NL754. G, T2 segregating progeny of a self-fertilized TAG850 T1 AdoMetDC-overexpressing plant showing the increased severity of the transgenic phenotypes compared with the T1 generation shown in B. A syngenic plant is to the left and transgenic plants displaying varying severities of phenotype to the right. H, flower phenotype typical of all the AdoMetDC-overexpressing plants with the transgenic flower to the right and syngenic to the left (these flowers from TAG850 T1, segregating progeny). I, leaf discs of segregating T1 NL754 progeny after 6 weeks on MS solid medium without hormones (transgenic discs above, syngenic discs below).
downstream of the original small uORF UGA termination codon. The region immediately downstream of the termination codon of sequence-independent uORFs is critical to their translationally repressive function (31, 32). Thus a construct that precisely alters the reading frame of the small uORF within the original boundaries of the uORF is needed to address the question of sequence-dependence.

The second line of evidence implicating the small uORF in the translational regulation of AdoMetDC comes from expression of wild type and mutant forms of the Arabidopsis AdoMetDC1 cDNA in transgenic tobacco plants. Removing the AdoMetDC 5′ leader sequence or C-terminal truncating the small uORF resulted in large increases in relative translational efficiency of the AdoMetDC ORF and in AdoMetDC activity compared with plants overexpressing the wild type cDNA. Overexpression of wild type AdoMetDC cDNA did not result in significantly increased AdoMetDC activity suggesting a translational homeostatic mechanism. However, the translationally derepressed AdoMetDC causes increased enzyme activity resulting in severe growth inhibition and morphological abnormalities. In both T1 and T2 generations, overexpression of AdoMetDC through translational deregulation resulted in depletion of putrescine and a 2–3-fold decrease in spermine but spermidine levels were more or less maintained at normal levels. The adaptive response of the plant to excess AdoMetDC activity was to prevent accumulation of more spermidine and spermine, suggesting that excess polyamines are deleterious to growth and normal cellular physiology. This reduction in spermine levels is in contrast to the response seen when AdoMetDC was overexpressed in mouse breast cancer cell lines (33) and to that seen with AdoMetDC gene amplification in

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**TABLE II**

Leaf polyamine content of T1 AdoMetDC-overexpressing plants

| Plant line | Putrescine | Spermidine | Spermine |
|------------|------------|------------|----------|
| 754N       | 279 ± 5.8  | 368 ± 1.90 | 25.3 ± 0.4 |
| 754T       | 14.4 ± 1.8 | 384 ± 4.44 | 13.8 ± 3.6 |
| 756N       | 261 ± 1.5  | 352 ± 6.50 | 35.1 ± 1.4 |
| 756T       | 21.9 ± 0.0 | 163 ± 16.7 | 8.9 ± 1.8  |
| 850N       | 184 ± 5.9  | 322 ± 23.1 | 31.6 ± 4.3 |
| 850T       | 13.1 ± 0.5 | 287 ± 22.4 | 13.0 ± 1.5 |

**TABLE III**

Leaf DNA 5′-methylcytosine as a percentage of total cytosine

Values represent the means of duplicate samples of pools of 16 transgenic (T) and 16 syngenic (N) T1 generation plants.

| Plant line | ((5′-mdC/5-mdC+dC)× 100) |
|------------|---------------------------|
| 754N       | 26.3 ± 2.4                |
| 754T       | 30.8 ± 3.3                |
| 756N       | 29.0 ± 2.6                |
| 756T       | 28.6 ± 1.1                |
| 850N       | 33.3 ± 4.3                |
| 850T       | 34.7 ± 6.4                |

**TABLE IV**

AdoMetDC activity in leaves of T1 segregating progeny of NL756 and TAG850 AdoMetDC-overexpressing plants

Values represent the AdoMetDC activities of independent pools of single leaves from independent plants for a total of 755 plants.

| Plant pools | Number of plants per pool | AdoMetDC activity nmol CO2/mg protein |
|-------------|---------------------------|--------------------------------------|
| 756N1       | 78                        | 0.164 ± 0.00                         |
| 756T1       | 114                       | 0.184 ± 0.10                         |
| 756N2       | 21                        | 0.195 ± 0.74                         |
| 756T2       | 24                        | 0.093 ± 0.07                         |
| 756N3       | 45                        | 0.020 ± 0.01                         |
| 756T3       | 73                        | 0.116 ± 0.66                         |
| 850N1       | 62                        | 0.133 ± 0.02                         |
| 850T1       | 90                        | 0.045 ± 0.00                         |
| 850N2       | 60                        | 0.051 ± 0.00                         |
| 850T2       | 69                        | 0.285 ± 0.02                         |
| 850N3       | 33                        | 0.52 ± 0.74                          |
| 850T3       | 86                        | 0.49 ± 0.10                          |

**TABLE V**

Leaf polyamine content of T2 AdoMetDC-overexpressing plants

Values represent three independent pools of transgenic (T) and syngenic (N) plants and are the means of duplicate dansyl chloride derivatizations and duplicate HPLC column injections. The plant pools are the same as those described in Table IV.

| Plant pool | Putrescine | Spermidine | Spermine |
|------------|------------|------------|----------|
| 756N1      | 108.8 ± 2.5| 306.8 ± 6.0| 19.8 ± 1.1|
| 756T1      | ND         | 390.8 ± 4.6| 14.5 ± 1.1|
| 756N2      | 122.3 ± 1.1| 318.5 ± 0.7| 23.0 ± 0.0|
| 756T2      | ND         | 311.3 ± 18.0| 10.5 ± 1.4|
| 756N3      | 070.0 ± 4.2| 218.5 ± 5.7| 16.0 ± 0.7|
| 756T3      | 004.5 ± 0.7| 336.5 ± 5.6| 11.5 ± 0.7|
| 850N1      | 495.0 ± 29  | 398.8 ± 18.0| 30.8 ± 1.1|
| 850T1      | ND         | 267.5 ± 29.7| 07.3 ± 1.0|
| 850N2      | 489.8 ± 3.9| 347.8 ± 5.3| 27.8 ± 1.1|
| 850T2      | 004.3 ± 3.6| 294.5 ± 27.6| 07.5 ± 1.4|
| 850N3      | 508.0 ± 1.3| 312.9 ± 1.5| 29.5 ± 7.8|
| 850T3      | 002.5 ± 0.0| 385.8 ± 8.1| 09.8 ± 1.1|

* 5′mdC, 5′-methyldeoxycytosine; dC, deoxycytosine.
Chinese hamster ovary cells (34), where most spermidine was converted to spermine. In the AdoMetDC-overexpressing plants the reduction in spermine levels is partly achieved by reduction of ornithine and arginine decarboxylase activities but it will also be necessary to analyze the spermidine and spermine synthase activities and the levels of ornithine and arginine amino acids as additional contributory factors controlling spermine accumulation.

The massive accumulation of decarboxylated AdoMet detected in the AdoMetDC-overexpressing plants could in principle cause severe problems for the plant cell. Decarboxylated AdoMet cannot act as a methyl donor but when present at high enough concentrations may act as a competitive inhibitor of DNA methyltransferase reactions (35). Indeed, derepression of AdoMetDC in F9 teratocarcinoma stem cells because of inhibition of ornithine decarboxylase activity resulted in a 30-fold increase in decarboxylated AdoMet levels and was associated with a decrease of cytosine methylation (36). However, caution is required when interpreting these results as the inhibition of ornithine decarboxylase caused the F9 cells to differentiate and the DNA demethylation observed was similar in extent to that observed when the same cells differentiated after retinoic acid treatment (36). Much higher levels of decarboxylated AdoMet (several hundred-fold) were observed after inhibition of ornithine decarboxylase in transformed mouse fibroblasts (37) and in rat hepatoma tissue culture cells (38). In the untreated F9 cells the decarboxylated AdoMet content was about 2–4% of the AdoMet content, similar to the figure of 2.5% that we determined in the T2 syngenic tobacco plants. The increase in the decarboxylated AdoMet content of the transgenic plants was exacerbate by the suppression of the aminopropyl-accepting putrescine. With no aminopropyl acceptor, the decarboxylated AdoMet could not be further metabolized through the 5'-methylthioadenosine route to the methionine salvage pathway (39).

The growth and morphological abnormalities of the AdoMetDC-overexpressing plants, especially the increasing severity of the phenotype in advancing generations is reminiscent of the Arabidopsis ddm1 mutant that causes a 70% decrease in methylated cytosine content (27, 40). It was also observed with the ddm1 mutant plants that there was variability of the morphological phenotype and severity among siblings in advanced generations (27): this is similar to the variable level of phenotype severity observed among the AdoMetDC-overexpressing siblings. The association of methylated cytosine depletion upon AdoMetDC derepression and decarboxylated AdoMet accumulation in F9 teratocarcinoma stem cells (36) and the similarity of the growth phenotypes of the Arabidopsis ddm1 mutant and the AdoMetDC-overexpressing tobacco plants led us to the obvious conclusion that the overexpression of AdoMetDC might result in methylated cytosine depletion. However, there was no detectable difference in total methylated cytosine content of the AdoMetDC-overexpressing plants. It is possible that the increasing severity of the phenotype is because of the increasing suppression of putrescine and reduction in spermine content in successive generations. The temperature sensitivity of the AdoMetDC-overexpressing tobacco plants is also reminiscent of the temperature sensitivity of polyamine-depleted yeast cells (41).

Perhaps the most surprising observation in the plants accumulating such high levels of decarboxylated AdoMet was that the concentrations of AdoMet were relatively unaffected, revealing the remarkable capacity of plants for buffering AdoMet levels. This is not specific to plants as overexpression of AdoMetDC because of gene amplification in Chinese hamster ovary cells resulted in more than a 200-fold increase in decarboxylated AdoMet content but AdoMet levels remained relatively unchanged (34). Such an efficient AdoMet homeostasis makes it unlikely that AdoMet-dependent processes such as transmethylation, ethylene biosynthesis, and threonine synthase allosteric activation are adversely affected directly. It would be surprising if the severe growth defects observed in the transgenic plants did not cause indirect stress-related changes to ethylene levels.

The stunting, leaf wrinkling, and delayed flowering of the transgenic plants are also reminiscent of tobacco plants treated with 2-mi α-methyldopa-ornithine, a substrate inhibitor of ornithine decarboxylase (42). It may be that the growth abnormalities because of overexpression of AdoMetDC are due paradoxically to an adaptive response by the plant that reduces polyamine levels. However, it is equally possible that the very high level of decarboxylated AdoMet is the main cause of the growth perturbations.

From this we can see that the plant AdoMetDC wild type mRNA overlapping uORF configuration is an extremely effective homeostatic mechanism for controlling AdoMetDC activity through regulation of the proenzyme translation. What remains to be shown is the relationship between the translational repression mechanism and polyamine-mediated post-transcriptional regulation of AdoMetDC. In addition, it will be necessary to identify the polyamine sensing mechanism.

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