Characterization and Functional Expression of cDNAs Encoding Methionine-sensitive and -insensitive Homocysteine S-Methyltransferases from Arabidopsis*

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Plants synthesize S-methylmethionine (SMM) from S-adenosylmethionine (AdoMet), and methionine (Met) by a unique reaction and, like other organisms, use SMM as a methyl donor for Met synthesis from homocysteine (Hcy). These reactions comprise the SMM cycle. Two Arabidopsis cDNAs specifying enzymes that mediate the SMM → Met reaction (SMM:Hcy S-methyltransferase, HMT) were identified by homology and authenticated by complementing an Escherichia coli yagD mutant and by detecting HMT activity in complemented cells. Gel blot analyses indicate that these enzymes, AtHMT-1 and -2, are encoded by single copy genes. The deduced polypeptides are similar in size (36 kDa), share a zinc-binding motif, lack obvious targeting sequences, and are 55% identical to each other. The recombinant enzymes exist as monomers. AtHMT-1 and -2 both utilize L-SMM or (S,S)-AdoMet as a methyl donor in vitro and have higher affinities for SMM. Both enzymes also use either methyl donor in vivo because both restore the ability to utilize AdoMet or SMM to a yeast HMT mutant. However, AtHMT-1 is strongly inhibited by Met, whereas AtHMT-2 is not, a difference that could be crucial to the control of flux through the HMT reaction and the SMM cycle. Plant HMT is known to transfer the pro-R methyl group of SMM. This enabled us to use recombinant AtHMT-1 to establish that the other enzyme of the SMM cycle, AdoMet:Met S-methyltransferase, introduces the pro-S methyl group. These opposing stereoselectivities suggest a way to measure in vivo flux through the SMM cycle.

Unlike other organisms, plants synthesize L-S-methylmethionine (SMM)1 from Met and S-adenosylmethionine (AdoMet) in a reaction mediated by AdoMet:Met S-methyltransferase (MMT, EC 2.1.1.12) (1–3). SMM can then serve as a methyl donor for the synthesis of Met from homocysteine (Hcy) catalyzed by Hcy S-methyltransferase (HMT, EC 2.1.1.10). The tandem action of MMT and HMT, plus that of S-adenosylhomocysteine (AdoHcy) hydrolase, constitutes the SMM cycle (Fig. 1). Although MMT and the SMM cycle are unique to plants, HMT occurs in bacteria, yeast, and mammals, enabling them to catabolize SMM of plant origin and providing an alternative to the methionine synthase reaction as a means to methylate Hcy (4–7).

In wheat and other plants, SMM is synthesized in leaves and transported via the phloem to developing seeds where it can be used to methylate Hcy (8). SMM is also synthesized by morning glory flower buds and then used to methylate Hcy during blooming (9). The halves of the SMM cycle can thus sometimes be separated in space or time. However, both halves may also operate concurrently in the same tissue, and in these cases the cycle has been hypothesized to remove excess AdoMet (3). Testing this hypothetical homeostatic role, which is analogous to that of the cyclic methylation/demethylation of Gly in mammalian liver (10), requires determination of flux through the SMM cycle in defined tissues in vivo. Methods to do this are lacking.

The first enzyme of the SMM cycle, MMT, has been purified from Wollastonia biflora and barley, and characterized (2, 11). MMT cDNAs have been isolated from W. biflora, Arabidopsis, and maize, and the two latter plants have been shown to have one MMT gene (8). Much less is known about plant HMTs, and none has been cloned from plants or other eukaryotes. HMT was partially purified from jack beans and germinating peas (12, 13) and shown to be stereoselective for one of the two methyl groups of SMM (the pro-R methyl) (14). The preparations obtained used either SMM or AdoMet as methyl donor; it was not established whether both activities reside on the same protein. These data appear to indicate that plants can bypass SMM by recycling AdoMet methyl groups directly to Met (Fig. 1, dotted arrows). However, the AdoMet substrates used in these experiments most probably contained significant levels of the nonphysiological R,S diastereomer (15), and it has been suggested that this, not the physiological S,S form, is the substrate for HMTs (7). The form of AdoMet that plant HMTs utilize is therefore unclear. Neither jack bean nor pea HMT

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank[e*]/EBI Data Bank with accession number(s) AF219222 (AtHMT-1) and AF219223 (AtHMT-2).

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‡ The abbreviations used are: SMM, S-methylmethionine; AdoMet,
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Separation of AdoMet Diastereomers—The S,S (biologically active) and R,S (inactive) diastereomers of AdoMet were separated by HPLC essentially as described by Beaudoin et al. (22). Analytical scale separations of [methyl-1-14C]AdoMet and unlabeled AdoMet were made on a 1 × 250 mm ReliaBead C18 column using a microbore HPLC system (Uma model, Michrom Bioresearches, Auburn, CA). Solvent A was water containing 0.1 M sodium acetate, 20 mM citric acid, 0.93 mM octanesulfonic acid, and 0.12 mM EDTA; solvent B was methanol, and the gradient was from 100–95% solvent A in 45 min. The elution profile was monitored at 258 nm. The [methyl-1-14C]AdoMet contained no detectable R,S form (1% or less) and was used without further purification. As was reported (15), unlabeled AdoMet was found to contain ~15% of the R,S isomer. In the few cases (see “Results”) in which unlabeled AdoMet was included in enzyme assays, specific radioactivity calculations were based on its (S,S)-AdoMet content.

E. coli and Saccharomyces cerevisiae Strains, Plasmids, and Growth Conditions—The E. coli strain used in complementation tests was MTD123 (aroD ΔmetE ΔmetD (16) and the expression vector was pBlueScript SK- (Stratagene). The minimal medium was M9 (24) containing 0.8% glucose, 1-Met, or 1-SMM (70 μM) and 1 μM isopropyl β-D-thiogalactopyranoside. The S. cerevisiae strains CY61–1A (MATα his3 leu2 ura3 ade2 trp1 met6::HIS3) and CY61–1D (MATα his3 leu2 ura3 ade2 trp1 met6::HIS3 ypl273::URA3 yll062::HIS3) were obtained from Y. Surdin-Kerjan (Centre de Génétique Moléculaire, CNRS, 91440 Gif-sur-Yvette, France). The yeast expression vector was pVT102-L (25). The synthetic minimal medium for yeast and the culture conditions were as described (26) except for the inclusion of adenine (100 μM) and 1-SMM or AdoMet (100 μM).

cDNA Generation, Sequencing, and Sequence Analysis—Arabidopsis expressed sequence tags, GenBank™ accession numbers T46013 and H37463 (encoding AtHMT-1 and -2, respectively), were obtained from the Arabidopsis Biological Resource Center (Columbus, OH). The ~750-bp base pair insert in H37463, which is truncated at the 5′-end, was used to isolate a full-length cDNA from an Arabidopsis (ecotype Landsberg erecta) leaf library in the λ Unizap XR vector (Stratagene) (provided by T. L. Thomas, Texas A&M University). DNA sequencing procedures were as described (8). Sequence alignments were made using Clustal W 1.7 (27). Phylogenetic analysis was carried out using the Dnuswim system at the ETH server. Homology searches were made using BLAST programs (28).

cDNA Expression in E. coli—HMT coding sequences were amplified from plasmid templates by high fidelity polymerase chain reaction using recombinant Pfu DNA polymerase (Stratagene). The primers included restriction sites for cloning into pBlueScript SK- and, for the forward primers, a Shine-Dalgarno sequence preceded by a stop codon in frame with the LacZ protein (forward), a Start codon with the lacZ transcriptional start site and the lacZ terminator sequence (reverse). After ligation into pBlueScript SK-, constructs were introduced into E. coli strain DH10B by electroporation. Plasmid preparations were sequenced to verify the inserts and used to transform E. coli strain MTD123 by electroporation.

cDNA Expression in Yeast—HMT coding sequences were amplified as above using primers that included the first or last eight codons plus restriction sites for cloning into pBlueScript SK- and, for the forward primers, a Shine-Dalgarno sequence preceded by a stop codon in frame with the LacZ protein (forward), a Start codon with the lacZ transcriptional start site and the lacZ terminator sequence (reverse). After ligation into pBlueScript, constructs were introduced into E. coli strain DH10B by electroporation. Plasmid preparations were sequenced to verify the inserts and used to transform E. coli strain MTD123 by electroporation.

Enzyme Isolation and Molecular Mass Determination—E. coli cultures (50 ml) were grown in an Axygen of 0.6–1 in LB medium (24) containing 100 μM ml–1 ampicillin and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; 0.5 mM) for 4 h. Cells were harvested by centrifugation and stored at −80°C. Subsequent operations were carried out at 0–10°C. Cells were resuspended in buffer A (5 ml/50 ml culture) and broken by sonication; the extract was cleared by centrifugation (10,000 × g, 15 min) and used for enzyme assays directly or after desalting on PD-10 columns (Amersharn).
Pharmacia Biotech) equilibrated in buffer A. Extracts were routinely stored at −80 °C after freezing in liquid N₂; this was shown not to affect HMT activity. Yeast extracts were prepared as described previously (26) using buffer A. Native molecular masses were estimated using a Waters 626 HPLC system equipped with a Superox 200 HR 10 × 25-cm column (Amerham Pharmacia Biotech); reference proteins were cytochrome c, carbonic anhydrase, bovine serum albumin, and β-amylase. Protein was estimated by Bradford's method (29) using bovine serum albumin as standard.

**Enzyme Assays**—Unless otherwise indicated, assays were made under conditions in which substrates were saturating and product formation was proportional to enzyme level and time. The assays were modified from those described by Mudie and Datko (3). SMM: Hcy S-methyltransferase assays (final volume 50 μl) contained 20 mM Hepes-KOH buffer, pH 7.5, 2 mM DTT, 2 mM Hcy (or other methyl acceptor), 200 μM [35S]SMM (15–25 nCi/assay), and enzyme extract. AdoMet: Hcy S-methyltransferase assays were similar except that the final volume was 5 μl and the Hepes-KOH concentration was raised to 200 mM (due to the H₂SO₄ in the [13C]SMM preparation); the [13C]SMM (yield 50%) was used as a substrate for HMT. The 1-ml reaction mixtures contained 10 mM potassium phosphate buffer, pH 7.5, 1 mM EDTA, 100 μm AdoMet, and 10 picokatal of recombinant Arabidopsis MMT activity. After incubation for 16 h at 30 °C, the mixture was passed through 1-ml Dowex-1 (OH⁻) and BioReX-70 (H⁺) columns arranged in series. Met, AdoMet, and AdoHcy were retained by the Dowex-1 column; the [35S]SMM (yield = 0.39 μmol) was eluted from BioReX-70 with 5 ml of 1 M HCl and lyophilized. The [35S]SMM was then used as a substrate for HMT. The 1-ml reaction mixtures contained 10 mM potassium phosphate buffer, pH 7.5, 1 mM DTT, 10% glycerol, 0.39 μmol of [35S]SMM, 1.5 μmol of Hcy, and desalted E. coli extract containing 2 nanokatal of AtHMT-1 activity. After incubation for 4 h at 30 °C, the mixture was passed through 1-ml columns of Dowex-50 (NH₄⁺), and Dowex-50 (H⁺) arranged in series. Residual SMM was retained on the first column; the [35S]Met product (yield = 0.35 μmol) was eluted from the second column with 5 ml of 6 M NH₄OH and lyophilized. The [35S]Met was separated from peptides and glycoprotein in the lyophilize by extraction in 95% ethanol and dried in vacuo.

**Electrospray Mass Spectrometry**—The [35S]Met formed by the sequential action of MMT and HMT was analyzed on a Finnigan MAT LCQ (Thermoquest, San Jose, CA) mass spectrometer system. The source voltage was set at 3.5 kV and capillary voltage at 30 V; the capillary temperature was 22 °C. Background source pressure was ~1.5 × 10⁻⁵ torr as read by an ion gauge. The sample flow rate was 10 μl min⁻¹. The drying gas was N₂. The LCQ was scanned to 2000 atomic mass units. Spectra were acquired for 0.5 min. Samples were dissolved in 50 μl of water; 25 μl was injected into the mass spectrometer.

**DNA Gel Blot Analyses**—Arabidopsis genomic DNA was isolated from leaves as described (30). Five-μg samples of the isolated DNA were digested, separated in 0.7% agarose gels, and transferred to supported nitrocellulose membrane (Nitrotype, MSQ) as described (24). Blots were hybridized overnight at 58 °C in 5× SSC, 5× Denhardt's solution, 1% SDS, 1 mm EDTA, and 100 μg ml⁻¹ sonicated salmon sperm DNA and washed at low stringency (1× SSC, 0.1% SDS, 37 °C). The probes were the full-length AtHMT-1 or -2 cDNAs and were labeled with 32P by the random primer method. Radioactive bands were detected by autoradiography.

**RESULTS**

**Genomic-based Cloning of HMT cDNAs from Arabidopsis**—BLAST searches using the amino acid sequence of *E. coli* YagD detected two sets of homologous Arabidopsis expressed sequence tags. Sequencing one insert from each set (GenBank access numbers T46013 and H37463) established that they represent two distinct transcripts. The T46013 insert encodes a 326-residue (36.4 kDa) polypeptide, designated AtHMT-1. The H37463 insert encodes only the C-terminal part of a polypeptide-

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2 D. Thomas, A. Becker, and Y. Surdin-Kerjan, personal communication.
diastereomer and, in the assay conditions used (pH 7.5, 30 min), ≲0.3% (R,S)-AdoMet is expected to form by racemization (15). (R,S)-AdoMet therefore did not contribute significantly to the observed activities. Because SMM and (S,S)-AdoMet are substrates, $K_v$ values for l-Hcy were determined with both (Table II); fairly similar values were obtained with both methyl donors and with both enzymes. To screen for other potential methyl donors, unlabeled compounds were tested for their ability to inhibit methyl transfer from [35S]SMM when added to methyl donors, unlabeled compounds were tested for their ability to inhibit methyl transfer from [35S]SMM when added to methyl donors, unlabeled compounds were tested for the ability to grow on SMM or AdoMet (Fig. 5) (this type of experiment cannot be carried out in E. coli because it cannot absorb AdoMet). AtHMT-1 and -2 enabled growth on SMM to Hcy (14), we used recombinant AtHMT-1 to determine the diastereospecificity of MMT, the other enzyme of the SMM cycle. To do this, L-[U-13C5]Met and unlabeled AdoMet were used as substrates for recombinant Arabidopsis MMT; the product of the MMT/HMT reactions gave peaks of almost equal intensity at m/z 151 and 154, corresponding to [13C1]Met and [13C5]Met.

**FIG. 2.** Alignment of the deduced amino acid sequences of *Arabidopsis* HMTs with related methyltransferases from other organisms. Identical residues are shaded in black, and similar residues are gray. Dashes are gaps introduced to maximize alignment. The bar indicates the core of the zinc-binding motif reported in B$_12$-dependent Met synthase and betaine-Hcy methyltransferase (18, 19). The asterisk marks a third conserved cysteine residue. AtHMT-1 and -2, *Arabidopsis* HMT-1 and -2; YagD, *E. coli* YagD (BA12002); Y1062c, *S. cerevisiae* Y1062c (S50958); Yp1273w*, *S. cerevisiae* Yp1273w (S65306); SecysMT, *A. bisulcatus* selencysteine S-methyltransferase (CAA10388).

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AtHMT-1 and -2 were expressed in *E. coli* strains Ypl273w (S65306); Yll062c (S50958); Yp1273w*, *S. cerevisiae* Yp1273w (S65306); SecysMT, *A. bisulcatus* selencysteine S-methyltransferase (CAA10388).

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in the original [13C5]Met substrate (Fig. 6). These data show that MMT introduces a methyl group into the pro-S position of SMM, i.e., that MMT and HMT have opposite stereoselectivities.

Genomic Complexity and Relationships of HMT Genes in Arabidopsis—Southern blot analyses carried out at low stringency indicated that both AtHMT-1 and AtHMT-2 are encoded by single genes (Fig. 7, A and B). Consistent with this result, BLAST searches of the Arabidopsis genome (~84% complete at the time of searching) revealed a chromosome III sequence specifying AtHMT-1 (AB023041, nucleotides 21893–23610) but no other closely related sequences. Molecular phylogenetic analysis (Fig. 7C) of the sequences aligned in Fig. 2 suggests (a) that AtHMT-2 and Astragalus SecysMT belong on a branch that extant HMTs are derived from a single ancestral gene that existed prior to the divergence of eubacteria and eukaryotes and has undergone independent duplications in plant and yeast lineages.

**TABLE I**

| Methyl acceptor | Methylation activity | AtHMT-1 | AtHMT-2 |
|-----------------|----------------------|---------|---------|
|                 | nmol min⁻¹ mg⁻¹ protein |         |         |
| l-Homocysteine  | 114.5 ± 4.0           | 12.6 ± 0.2 |         |
| d-Homocysteine  | 20.1 ± 1.5            | 10.8 ± 0.2 |         |
| L-Cysteine      | 3.3 ± 0.9             | <0.05    |         |
| d-Cysteine      | 3.4 ± 0.2             | <0.05    |         |
| L-Selenocysteine| <0.05                 | <0.05    |         |

**TABLE II**

| Variable substrate | Constant substrate | AtHMT-1 | AtHMT-2 |
|--------------------|--------------------|---------|---------|
|                    | K_m (μM) | V_max (μM) | K_m (μM) | V_max (μM) |
| l-SMM              | 29       | 1*       | 50       | 1b       |
| (S,S)-AdoMet       | 1950     | 0.36     | 225      | 0.55     |
| l-Hcy              | 67       | 14       | 18       | 1        |
| (S,S)-AdoMet       | 74       | 1        | 37       |          |

* V_max = 139 nmol min⁻¹ mg⁻¹ total protein.
** V_max = 15.9 nmol min⁻¹ mg⁻¹ total protein.

Unlabeled AdoMet was used in assays in which total [AdoMet] was 200 μM or above.
DISCUSSION

The identification of cDNAs encoding plant HMTs completes the set of genes required for operation of the SMM cycle, the others being MMT and AdoHcy hydrolase (3). This opens the way to comprehensive studies of the expression of these genes and to the systematic application of reverse genetics to probe the function of SMM and its cycle. Furthermore, extracts of E. coli expressing AtHMT-1 or -2 have specific activities $10^2$-fold higher than those of the best plant sources (12, 13) making them good material for future enzyme purification. More generally, the HMT cDNAs reported here appear to be the first identified from a eukaryote.

AtHMT-1 and -2 resemble HMTs from other organisms in overall primary structure and in being monomeric proteins. They lack obvious targeting sequences and are therefore presumably cytosolic enzymes. HMT has yet to be definitively localized in plant cells, but preliminary work with pea leaves indicates that it is cytosolic, as are other enzymes involved in Met metabolism, i.e., MMT, Met synthase, AdoMet synthetase, and AdoHcy hydrolase (8, 32). AtHMT-1 and -2 share with other HMTs and with SecySMT, a GGCC zinc-binding motif (18), plus a third conserved cysteine residue. This strongly suggests that they have a zinc cofactor. Neither enzyme was stimulated by zinc or severely inhibited by EDTA, but this may be because the zinc is tightly bound, as it is in betaine-Hcy methyltransferase (19).

Our results demonstrate that the physiological $S,S$ diastereomer of AdoMet is a substrate for plant HMTs. This indicates that plants have the potential to bypass SMM by transferring methyl groups directly from AdoMet to Hcy (Fig. 1, dotted arrows), and the complementation experiments with yeast confirm that plant HMTs can mediate this reaction in a foreign host. But how much flux does this bypass actually carry in planta? Kinetic considerations indicate that it may be very little, especially in tissues where AtHMT-1 is the predominant isoform. AtHMT-1 has $K_m$ values for SMM and AdoMet of 29 and 1950 $\mu$M, respectively, and the $V_{\text{max}}$ value with SMM is 2.8-fold higher. SMM levels are reported to range from about 5 to $>300$ nmol g$^{-1}$ fresh weight in various tissues, and SMM/AdoMet ratios are reported to range from $\sim$1 to $>30$ (1, 8, 33–36). Some SMM may be sequestered in the vacuole; however, radiotracer kinetic studies indicate that the metabolically active (presumably cytosolic) SMM pool is a large fraction of the total (36). Assuming the cytosol to be $\sim$5% of tissue water volume (37), it follows from these data that typical cytosolic SMM concentrations are likely to be $>100$ $\mu$M, and AdoMet concentrations are likely to be similar or lower. In such conditions flux through the AdoMet-driven reaction would be $\sim$3% of that through the SMM-driven reaction. Simply put, a high prevailing SMM concentration can deny AdoMet access to the AtHMT-1 active site and thereby suppress futile cycling of AdoMet.

Our finding that AtHMT-1 is strongly inhibited by Met is...
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novel, because the plant HMTs so far known are Met-insensitive (12, 13). Met sensitivity may be crucial to the control of flux through the HMT reaction and the SMM cycle. A Met-sensitive HMT could stop the cycle turning when Met levels are elevated, whereas a Met-insensitive enzyme could allow SMM → Met conversion even when Met levels are high. It is therefore noteworthy that free Met levels in developing seeds can greatly exceed those in other tissues (≥400 versus 10–30 nmol g⁻¹ fresh weight) (1, 35, 38–40) and that HMTs isolated from seeds are Met-insensitive (12, 13). Moreover, DNA array data indicate that the predominant HMT expressed in developing Arabidopsis seeds is the Met-insensitive AtHMT-2.4 Another difference between the Arabidopsis HMTs is that AtHMT-1 attacks cysteine. This could explain the origin of S-methylcysteine in the Brassicaceae. No enzyme that catalyzes the S-methylation of cysteine has hitherto been demonstrated (1), although radiotracer data show that the reaction occurs in vivo (41).

The SMM cycle has been proposed to rectify overshoots in the conversion of free Met to AdoMet, thereby sustaining a free Met pool for protein synthesis (3). This hypothesis was based largely on data for whole Lemma plantlets (3), and it has since been found that SMM is transported between organs in the phloem (8). This raises the question of whether the SMM was produced and utilized in the same organs in the Lemma experiments and shows that accurate flux measurements are now needed to clarify the functions of the SMM cycle. Only a few such measurements have been made, and these come from unusual plants (W. biflora and Spartina alterniflora) that convert SMM to DMS. Isotope tracer studies of SMM synthesis and metabolism in leaves of these plants showed that the methyl flux from Met to SMM was high, but there was little or none from SMM to Hcy, i.e. the SMM cycle turned slowly if at all (36, 42). The approach used to make these measurements depends on the metabolism of SMM to DMS and so unfortunately cannot be applied to the great majority of plants that do not synthesize DMS.

There is thus a need for methods to estimate flux through the SMM cycle in tissues of non-DMS-accumulating plants. Our finding that the enzymes of the cycle have opposing stereoelectivities suggests a novel way to do this. For example, consider an organ that imports SMM via the phloem and ultimately uses it to produce Met that is used for protein synthesis. If the SMM cycle is not operating, then supplied SMM that has a 13C label in the C5 backbone and the pro-R methyl and 2H3 label in the pro-S methyl will give rise to only two labeled species of Met in proteins: [methyl-2H3,13C4]Met and [methyl-13C5]Met. However, if the SMM cycle is operating, the additional species [methyl-2H3]Met, [13C2]Met, and [13C5]Met will be found in proteins and will become relatively more abundant with each turn of the cycle.

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