

S-Methylmethionine Plays a Major Role in Phloem Sulfur Transport and Is Synthesized by a Novel Type of Methyltransferase

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All flowering plants produce S-methylmethionine (SMM) from Met and have a separate mechanism to convert SMM back to Met. The functions of SMM and the reasons for its interconversion with Met are not known. In this study, by using the aphid stylet collection method together with mass spectral and radiolabeling analyses, we established that L-SMM is a major constituent of the phloem sap moving to wheat ears. The SMM level in the phloem (~2% of free amino acids) was 1.5-fold that of glutathione, indicating that SMM could contribute approximately half the sulfur needed for grain protein synthesis. Similarly, L-SMM was a prominently labeled product in phloem exudates obtained by EDTA treatment of detached leaves from plants of the Poaceae, Fabaceae, Asteraceae, Brassicaceae, and Cucurbitaceae that were given L-³⁵S-Met. cDNA clones for the enzyme that catalyzes SMM synthesis (S-adenosylMet:Met S-methyltransferase; EC 2.1.1.12) were isolated from *Wollastonia biflora*, maize, and *Arabidopsis*. The deduced amino acid sequences revealed the expected methyltransferase domain (~300 residues at the N terminus), plus an 800-residue C-terminal region sharing significant similarity with aminotransferases and other pyridoxal 5'-phosphate-dependent enzymes. These results indicate that SMM has a previously unrecognized but often major role in sulfur transport in flowering plants and that evolution of SMM synthesis in this group involved a gene fusion event. The resulting bipartite enzyme is unlike any other known methyltransferase.

INTRODUCTION

Plant Met metabolism differs from that in other organisms by involving S-methylmethionine (SMM). SMM is a ubiquitous constituent of the free amino acid pool in flowering plants, occurring in leaves, roots, and other organs at levels that typically range from 0.5 to 3 $\mu\text{mol g}^{-1}$ dry weight, a concentration that is often higher than those of Met or S-adenosylmethionine (AdoMet) (Giovanelli et al., 1980; Mudd and Datko, 1990; Bezzubov and Gessler, 1992). SMM also has been detected as a metabolite of radiolabeled L-Met in all flowering plants tested (>50 species from >20 families; Paquet et al., 1995). As shown in Figure 1, SMM is formed from L-Met via the action of AdoMet:Met S-methyltrans-

ferase (MMT; EC 2.1.1.12) and can be reconverted to Met by donating a methyl group to L-homocysteine (Hcy) in a reaction catalyzed by Hcy S-methyltransferase (HMT; EC 2.1.1.10; Giovanelli et al., 1980; Mudd and Datko, 1990). The tandem action of MMT and HMT, together with S-adenosyl-L-Hcy hydrolase, constitutes the SMM cycle, which is apparently futile (Mudd and Datko, 1990).

As expected from the universality of SMM, MMT activity has been found in many flowering plants (Giovanelli et al., 1980; Mudd and Datko, 1990). It has been purified from leaves of *Wollastonia biflora* (James et al., 1995a) and from germinating barley (Pimenta et al., 1998), and it is known to have subunits of ~115 kD. Because this is approximately three times larger than any other small-molecule methyltransferase (Fujioka, 1992; Joshi and Chiang, 1998), it suggests that the MMT polypeptide could have more than one

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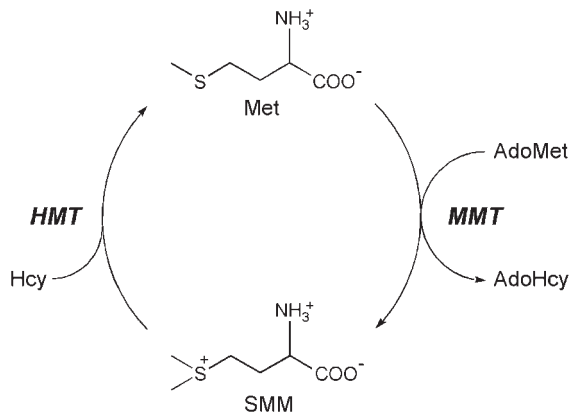


Figure 1. The SMM Cycle of Flowering Plants.

The reaction mediated by HMT generates two molecules of Met. AdoMet, S-adenosyl-L-homocysteine.

enzymatic domain. HMT has been less investigated, but it too has been found in diverse plants, especially in seeds (Giovannelli et al., 1980).

Although it is clear how plants synthesize SMM and reconvert it to Met, the physiological roles of SMM and its cycle remain undefined, except for the few species that use SMM as the precursor for synthesis of the osmoprotectant 3-dimethylsulfoniopropionate (Trossat et al., 1996; Kocsis et al., 1998). Proposed general roles for SMM and its cycle include Met storage, methyl donation, and regulation of the AdoMet/Met ratio, all of which are reasonable but unsupported by experimental evidence (Giovannelli et al., 1980; Mudd and Datko, 1990). In all of these proposed roles, SMM would exert its functions without exiting the cells that produce it.

Data reported in a study of sieve tube protein turnover by Fisher et al. (1992) led us to suspect that SMM might have a quite different role—in phloem sulfur transport. Fisher et al. (1992) found that when wheat flag leaves were given ^{35}S -Met, most of the sulfur-35 in the phloem sap moving to the ear was in an unidentified metabolite with low mobility in thin-layer chromatography (TLC). We noted that this product had the mobility characteristic of SMM. In this study, we repeated the experiment of Fisher et al. (1992) and demonstrated that the unidentified phloem-mobile metabolite was indeed ^{35}S -SMM. This prompted us to quantify the SMM present in wheat phloem by mass spectral methods and to determine whether SMM occurs in the phloem sap of other flowering plants. Because the data indicated that SMM is a major and common phloem constituent, we proceeded to isolate and characterize cDNAs for MMT, the SMM-synthesizing enzyme, from three diverse plants. The deduced MMT amino acid sequences define a novel type of methyltransferase.

RESULTS

Wheat Leaves Supplied with ^{35}S -Met Export ^{35}S -SMM in the Phloem

Using the same procedures as Fisher et al. (1992), we gave a flag leaf from a wheat plant in the middle part of the grain-filling period a pulse of tracer $\text{L-}^{35}\text{S}$ -Met and collected phloem exudate from the peduncle by using severed aphid stylets (Figure 2A). Analysis of the soluble labeled compounds from both exudate and leaf showed that SMM is a prominent metabolite, accounting for $\sim 80\%$ of the total sulfur-35 in the exudates and 60% in the leaves (Figure 2B). The identity of ^{35}S -SMM was established by three criteria: comigration with authentic SMM in three separation systems; complete destruction by hot 1 M NaOH; and conversion to the corresponding α -hydroxy acid by nitrous acid treatment (Figure 2C). The proportion (80%) of the total sulfur-35 in the exudate that was accounted for by ^{35}S -SMM matched that reported for the unidentified ^{35}S -Met metabolite (i.e., SMM) by Fisher et al. (1992).

Mass Spectral Evidence That SMM Is a Major Form of Sulfur in Wheat Phloem Sap

To reinforce the radiolabeling evidence for the presence of SMM in wheat phloem, we analyzed exudates by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). There was a strong signal at a mass-to-charge ratio (m/z) of 164, corresponding to SMM (Figure 3A). Confirmation that this peak represented SMM was obtained by MALDI postsource decay experiments; these showed the expected fragment at an m/z ratio of 102, formed from the precursor ion (m/z of 164) by neutral loss of $(\text{CH}_3)_2\text{S}$ (Figure 3B). MALDI-MS also was used to quantify SMM in wheat phloem exudates by using an internal standard of methyl- $^2\text{H}_6$ -SMM; the free α -amino acid contents of same samples were determined using ninhydrin. As shown in Table 1, SMM made up 1.8 ± 0.1 mol% (mean \pm SE) of free amino acids. This value suggested that SMM import could be a major item in the sulfur budget of the grain, given that the sulfur amino acid content of wheat grain protein is ~ 4 mol% (Khan and Eggum, 1978). The SMM level in flag leaf blades was measured for comparison with the phloem data; it was 170 ± 20 nmol g^{-1} fresh weight (mean \pm SE, $n = 3$) or ~ 85 nmol per blade.

SMM Levels in Wheat Phloem Sap Are Similar to Glutathione Levels

Because glutathione (GSH) is known to be a major form of reduced sulfur in the phloem of many species (Rennenberg, 1982; Brunold and Rennenberg, 1997), we compared the

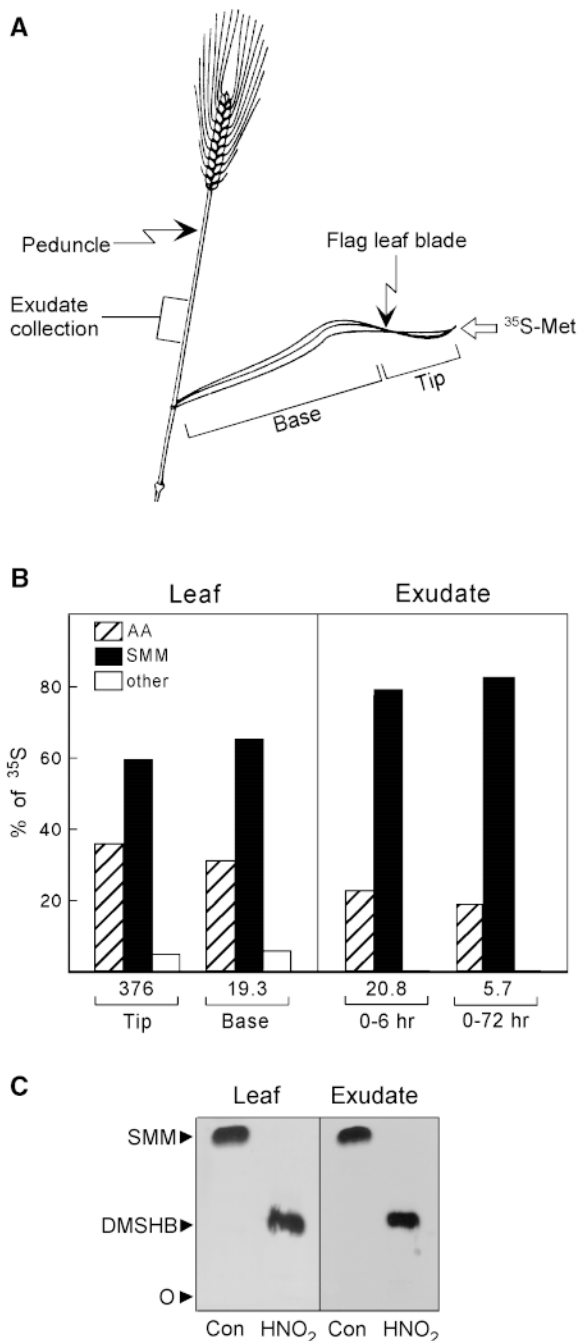


Figure 2. Export of ^{35}S -SMM from the Wheat Flag Leaf via the Phloem.

(A) Diagram showing the sites of ^{35}S -Met application and stylet exudate collection. A tracer dose of ^{35}S -Met (1 mCi, 1 nmol) was applied to the cut end of the flag leaf. The 2-cm terminal section of the leaf (Tip) was harvested 3 hr later; the remainder of the flag leaf blade (Base) was harvested 3 hr later; the remainder of the flag leaf blade (Base) was harvested 72 hr. Phloem exudate was collected in two fractions, 0 to 6 hr and 6 to 72 hr.

(B) The distribution of ^{35}S between SMM, the neutral plus acidic amino acid fraction (AA), and other water-soluble metabolites (other)

levels of GSH and related thiols to those of SMM. For these experiments, the phloem samples were collected at 40-min intervals and immediately frozen because tests indicated that the thiols in exudates underwent slow degradation in air at room temperature ($\sim 50\%$ loss in 14 hr). Table 2 shows that the GSH levels in wheat phloem were $66 \pm 3\%$ (mean \pm SE) of SMM levels. The GSH precursor γ -glutamylcysteine was present in lesser amounts, as was a compound with the chromatographic properties of γ -glutamylcysteinylserine. This homolog of GSH has been found in wheat leaves at levels ranging from three- to 15-fold lower than those of GSH itself (Klapheck et al., 1992; McKee et al., 1997), and its level also varied markedly among the phloem exudate samples. As reported previously for wheat phloem (Fisher and Macnicol, 1986), free Cys was virtually absent. The total thiol level was similar to the SMM level (Table 2).

Leaves of Diverse Flowering Plants Export SMM in the Phloem

To determine whether SMM is a constituent of phloem sap in other species, we used EDTA to enhance phloem exudation from cut leaf bases or petioles. EDTA chelates the Ca^{2+} required for callose formation and thereby blocks the sealing of cut sieve tubes (King and Zeevaart, 1974). The sap obtained by the EDTA technique is comparable in composition to that from severed stylets (Weibull et al., 1990; Valle et al., 1998). A tracer dose of ^{35}S -Met was applied to the tips of leaves from species representing five diverse families, and the corresponding phloem exudates were analyzed for ^{35}S -SMM (Figure 4A). Amino acid exudation was measured from matching unlabeled leaves, plus or minus EDTA, as a check on the technique. EDTA enhanced amino acid exudation by an average of ninefold (Figure 4B), which is consistent with the exudates coming mainly from cut sieve tubes (King and Zeevaart, 1974). ^{35}S -SMM was detected in the exudates from all 11 species tested, and in five of them it accounted for $\geq 35\%$ of the total label (Figure 4A). The proportion of ^{35}S -SMM in the exudate from wheat (70%) agreed well with that seen in stylet-derived exudate (Figure 2B), which further

in flag leaf extracts and phloem exudates. The sulfur-35 in the AA fraction was mainly in Met and its sulfoxide (formed by chemical oxidation of Met). The total sulfur-35 activity (μCi) in each sample is shown below the bar graphs.

(C) Confirmation of the identity of ^{35}S -SMM isolated from flag leaf and phloem exudate by conversion to the corresponding α -hydroxy acid, 4-dimethylsulfonio-2-hydroxybutyrate (DMSHB), when treated with nitrous acid. The autoradiographs shown are of thin-layer electrophoresis separations (see Methods for details); the positions of authentic standards are indicated. Con, control not treated with nitrous acid; O, electrophoresis origin.

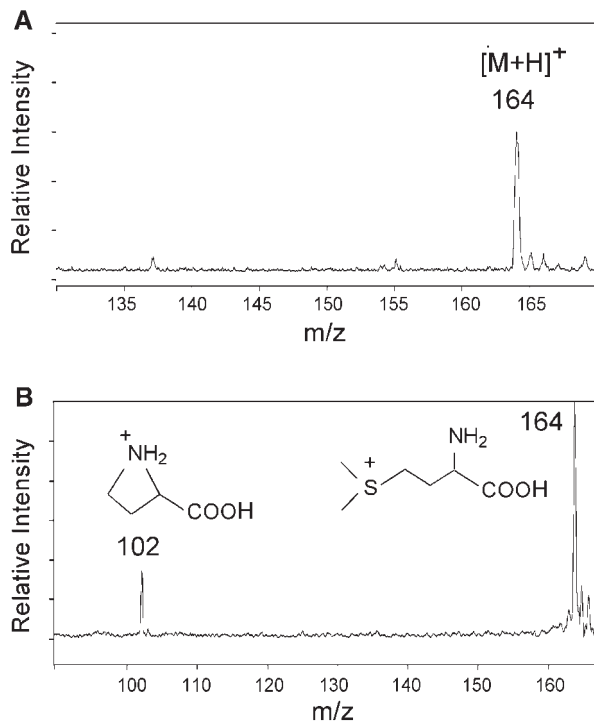


Figure 3. Mass Spectral Evidence for the Presence of SMM in Wheat Phloem Exudate.

(A) MALDI-MS spectrum of the BioRex-70 fraction. The major peak at m/z of 164 corresponds to SMM; the small peaks at m/z of 165 and 166 are due to natural-abundance C, S, and O isotopes.

(B) MALDI postsource decay experiment showing the predicted fragment ion at m/z of 102.

validates this application of the EDTA technique. In all our sulfur-35 exudation experiments, the applied tracer ^{35}S -Met may have entered the phloem directly, whereas sulfur-35 could only have reached SMM after isotope dilution by endogenous Met and SMM pools. These pools are far larger than the applied ^{35}S -Met doses and vary in size with species and leaf age (Gessler et al., 1991). Thus, the low proportions of ^{35}S -SMM in the exudates of some species (Figure 4A) by no means necessarily connote low chemical levels of SMM.

The SMM Present in Leaves and Phloem Sap Is Exclusively the L-Enantiomer

The enzyme that catalyzes SMM formation, MMT, is known to be specific for L-Met (James et al., 1995a), but the configuration of SMM itself has not been determined. We corrected this deficiency by analyzing ^{35}S -SMM isolated from leaves and phloem exudates of a monocot (wheat) and a dicot (Arabidopsis). We exploited the stereoselectivity of L- and D-amino acid oxidases, which convert SMM to an α -keto

acid that decomposes rapidly to yield dimethylsulfide (DMS; Rhodes et al., 1997). The relative amounts of ^{35}S -DMS released by the L- and D-specific enzymes showed that the ^{35}S -SMM from leaves and phloem of both species was essentially all ($\geq 97\%$) the L-enantiomer (Figure 5). This result establishes that SMM is not enzymatically racemized.

cDNA Cloning, Sequence Analysis, and Functional Expression of MMTs

The widespread occurrence of SMM in phloem sap led us to isolate cDNAs for the SMM-synthesizing enzyme, MMT, starting with *W. biflora*. Purified *W. biflora* MMT was digested to obtain peptides, 14 of which were sequenced. Degenerate polymerase chain reaction (PCR) primers matching the ends of a 19-residue peptide (see Methods) were used to amplify the corresponding 56-bp DNA sequence, with a *W. biflora* cDNA library as template. A primer specific for the central part of this 56-bp sequence, together with one from the vector, enabled PCR amplification of a 1.1-kb fragment comprising the 3' terminal region of MMT. This fragment was used to screen the library, which led to isolation of a 3.1-kb MMT cDNA that lacked the 5' region. The missing region was obtained by rapid amplification of cDNA ends (RACE). The complete cDNA encodes a 1088-residue protein of calculated mass of 121.6 kD (Figure 6). The deduced amino acid sequence includes all the MMT peptides that were sequenced, establishing its authenticity (Figure 6). No N-terminal signal sequence was recognizable, consistent with the exclusively cytosolic localization of *W. biflora* MMT (Trossat et al., 1996).

Searches of expressed sequence tag (EST) databases using the *W. biflora* nucleotide and amino acid sequences revealed matches with 16 maize and two Arabidopsis ESTs. The longest maize EST (3.4 kb) was sequenced and found to encode a 1091-residue protein with 62% amino acid identity (77% similarity) to *W. biflora* MMT (Figure 6). One Arabidopsis EST (1.2 kb) was sequenced, confirmed to encode a

Table 1. Levels of SMM and Total α -Amino Acids in Wheat Phloem Exudates^a

Experiment Number	Weight ^b (mg)	SMM (nmol)	α -Amino Acids (nmol)	SMM Level	
				Mol%	nmol mg ⁻¹
1	8.8	187	8750	2.14	21.3
2	10.8	215	12490	1.72	19.9
3	9.3	247	14270	1.73	26.6
4	32.0	383	25140	1.52	12.0

^aIn each experiment, phloem exudate was collected from one peduncle for 14 hr (experiments 1 to 3) or 72 hr (experiment 4).

^bFresh weight. The dry weight of exudates was $\sim 80\%$ of the fresh weight.

Table 2. Levels of Glutathione, Other Thiols, and SMM in Wheat Phloem Exudates^a

Experiment Number	α -Amino Acids	Cysteine	γ -EC	γ -ECS	GSH	SMM	Mol% (S/ α -Amino N)		
							GSH	Total Thiols	SMM
5	1686	0.08	1.25	4.70	17.0	24.4	1.01	1.37	1.44
6a	622	0.05	2.44	0.42	9.72	16.4	1.56	2.03	2.63
6b	634	0.09	1.07	0.34	10.1	13.8	1.59	1.82	2.18
6c	464	0.06	0.93	0.15	6.18	10.3	1.33	1.58	2.22
6d	381	0.07	1.25	0.13	5.77	8.65	1.51	1.90	2.27

^aIn both experiments, paired exudate samples (~1 mg each) were collected at 40-min intervals from three peduncles; one of each pair was analyzed for thiols, the other was analyzed for SMM and amino acids. In experiment 6, four pairs of samples (a, b, c, and d) were collected in succession from the same peduncles; samples a and b were collected just after stylets were severed, and c and d were collected the following day. All data, except the mol% values, are in units of nanomoles per milligram fresh weight of exudate. γ -EC, γ -glutamylcysteine; γ -ECS, γ -glutamylcysteinylserine.

polypeptide similar to *W. biflora* MMT, and used to screen an Arabidopsis cDNA library. This screen identified a clone with a 3.4-kb insert that encoded a 1071-residue protein with 67% amino acid identity (81% similarity) to *W. biflora* MMT (Figure 6).

The sequence context of the 3.4-kb Arabidopsis cDNA (in pBluescript SK-) indicated that the MMT was likely to be expressed in *Escherichia coli* in its native form, rather than as a LacZ fusion, owing to an in-frame stop codon sited just before the deduced initiation codon. Therefore, we extracted soluble proteins from *E. coli* cells harboring this clone and assayed MMT activity (Figure 7A). High activity was detected, the mean value (590 pmol min⁻¹ mg⁻¹ protein) being approximately threefold greater than the values reported for *W. biflora* and barley tissue extracts (James et al., 1995a; Pimenta et al., 1998). Cells harboring empty pBluescript KS+ vector had no MMT activity (Figure 7A), consistent with other evidence that *E. coli* lacks this enzyme (Neuhierl et al., 1999). We also transformed *E. coli* MTD123 (Thanbichler et al., 1999) with the Arabidopsis MMT clone or with pBluescript KS+, and tested the transformants for the ability to form ³⁵S-SMM from supplied ³⁵S-Met (Figure 7B). Strain MTD123 cannot catabolize SMM due to an in-frame deletion in the gene (*yagD*) that encodes HMT (Thanbichler et al., 1999). Cells expressing MMT accumulated a large amount of ³⁵S-SMM (13% of the total soluble sulfur-35), whereas control cells harboring the empty vector accumulated none (Figure 7B). These results establish that the MMT cDNA of Arabidopsis encodes the expected activity and also that MMT is metabolically functional in *E. coli*. Because the active MMT enzyme is a tetramer (James et al., 1995a), it can be further inferred that the subunit assembly process occurs with at least moderate efficiency in *E. coli* cells.

DNA gel blot analysis indicated that *MMT* is probably a single-copy gene in both Arabidopsis (Figure 8) and maize (data not shown), based on signal strength relative to genomic reconstructions and on the numbers of bands. The

two bands present in the HindIII and BglIII digests of Arabidopsis DNA are due to restriction sites in the coding region. Consistent with maize having only one *MMT* gene, no sequence diversity was found among 16 maize MMT ESTs.

MMTs Have Methyltransferase and Pyridoxal 5'-Phosphate Protein Domains

BLAST searches of the nonredundant protein database by using the MMT amino acid sequences yielded two significant sets of hits on different regions of the MMT polypeptide. As might be expected, one set comprised various methyltransferases; these hits occurred in the N-terminal 300 residues. Within this region, the section with the highest similarity to other methyltransferases (residues ~120 to 240; Figure 9A) included the consensus sequences for methyltransferase motifs I and post-I, which are involved in AdoMet binding (Kagan and Clarke, 1994; Gary et al., 1996). The MMTs also had correctly sited sequences similar to methyltransferase motifs II and III (Figure 9A); these generally are less conserved than motif I and sometimes absent, especially from plant methyltransferases (Kagan and Clarke, 1994; Joshi and Chiang, 1998). These results suggest that MMT has a discrete N-terminal methyltransferase domain of typical size (~300 residues). The sequence identity is greater among the three MMTs in this putative methyltransferase region (69% identity) than in the rest of the protein (47% identity; Figure 6).

The other significant BLAST hits fell in the last 400 residues of the C-terminal region and were to pyridoxal 5'-phosphate (PLP)-dependent enzymes, particularly group I aminotransferases and 1-aminocyclopropane-1-carboxylate (ACC) synthases. These enzymes belong to the α family of PLP-dependent proteins (Alexander et al., 1994; Jensen and Gu, 1996); in such proteins, the PLP prosthetic group is covalently bound to the ϵ -amino group of a specific lysine

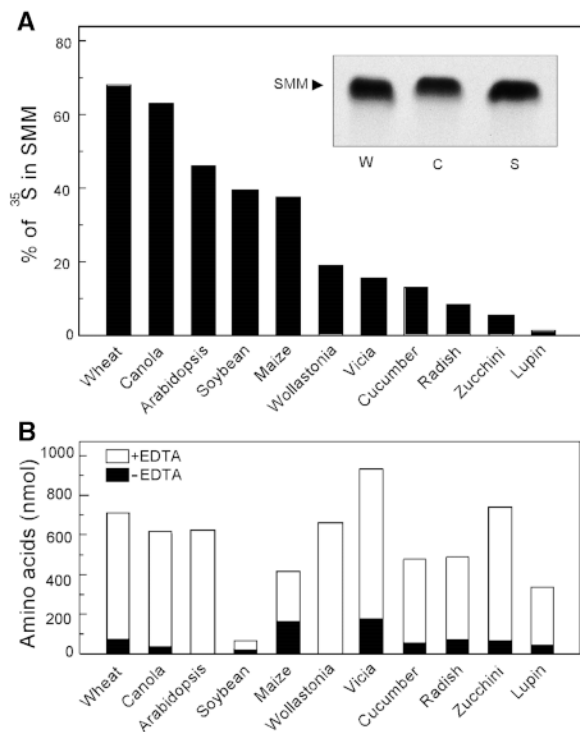


Figure 4. Evidence That SMM Occurs in Phloem Exudates from Diverse Plants.

(A) The ³⁵S-SMM contents of exudates, as a percentage of total sulfur-35 exuded. Attached leaves were supplied with 20 μ Ci (17 pmol) of L-³⁵S-Met, severed after 2 hr, and placed with their cut ends in 5 mM Na₂EDTA, pH 7.0, for 20 hr in darkness. Total sulfur-35 exudation ranged from 7.6 nCi (maize) to 917 nCi (Arabidopsis). Inset is an autoradiograph of a TLC separation of the BioRex-70 fraction of exudates from representative species: wheat (W), canola (C), and soybean (S).

(B) The levels of amino acids exuded with (+) or without (–) 5 mM Na₂EDTA in the medium. The leaves used were matched in size and age to those supplied with ³⁵S-Met. The values for zucchini have been multiplied by 0.5 to fit the scale used for other species.

residue. Alignments indicated that MMTs have three of the four invariant residues that characterize all aminotransferases, Gly-197, Asp/Glu-222, and Arg-386, yet lack the fourth and most crucial one, the PLP binding Lys-258 (Figure 9B; numbering is according to the sequence of the prototype, porcine cytosolic aspartate aminotransferase; Mehta et al., 1993). Other residues found in group I aminotransferases, Asn-194, Pro-195, Tyr-225, and Gly-268, are also present in MMTs or are conservatively replaced. Moreover, MMTs show 18 to 23% overall amino acid identity (36 to 42% similarity) to the α family enzymes in Figure 9B and

have similar hydropathy profiles, with the major exception of an \sim 20-residue hydrophobic insert that comprises the region in which the PLP binding lysine would be expected to reside (Figure 9C).

The C-terminal section of MMT thus has enough of the protein scaffold shared by aminotransferases and other α family proteins to suggest that it is descended from them, but it does not bind PLP in the usual site, if at all. Because MMTs have several conserved lysines in the C-terminal region (Figure 6), a highly displaced PLP binding lysine cannot be ruled out, although the conserved three-dimensional structure of α family enzymes makes this seem improbable (John, 1998). It is nevertheless noteworthy that the conserved lysine nearest the C terminus is set in a 40-residue sequence that is 23 to 35% identical (33 to 43% similar) to those around the PLP binding lysine in glucan phosphorylases, a group unrelated to the α family (John, 1998). Another possibility is that PLP is bound noncovalently to the MMT polypeptide, as in various engineered enzymes (John, 1998), but no natural instances of this are known.

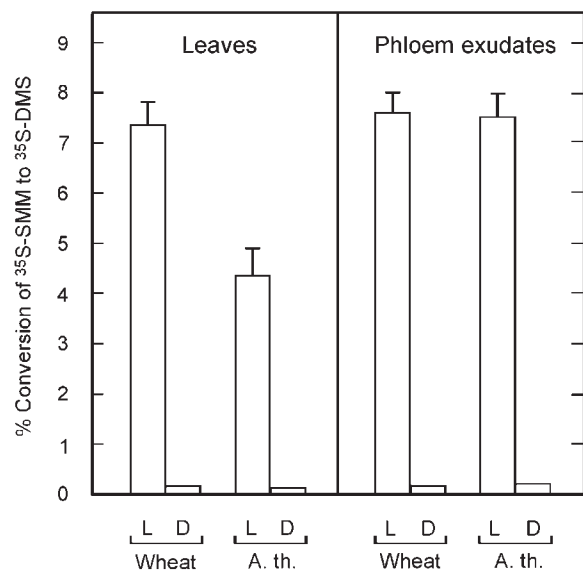


Figure 5. Determination of the Configuration of SMM from Leaves and Phloem.

³⁵S-SMM isolated from leaves or phloem exudates was incubated with L- or D-amino acid oxidase preparations of equivalent activity, and the ³⁵S-DMS reaction product was quantified. The relative amounts of ³⁵S-DMS released indicate in every case that 97 to 98% of the SMM was the L-enantiomer. Slight racemization during sample processing may account for traces of the D-form. Phloem exudates were obtained by the EDTA technique, except for one wheat sample from stylets. Data are means \pm SE of duplicate determinations on two independent samples. A. th., *Arabidopsis thaliana*; D, incubated with D-amino acid oxidase; L, incubated with L-amino acid oxidase.

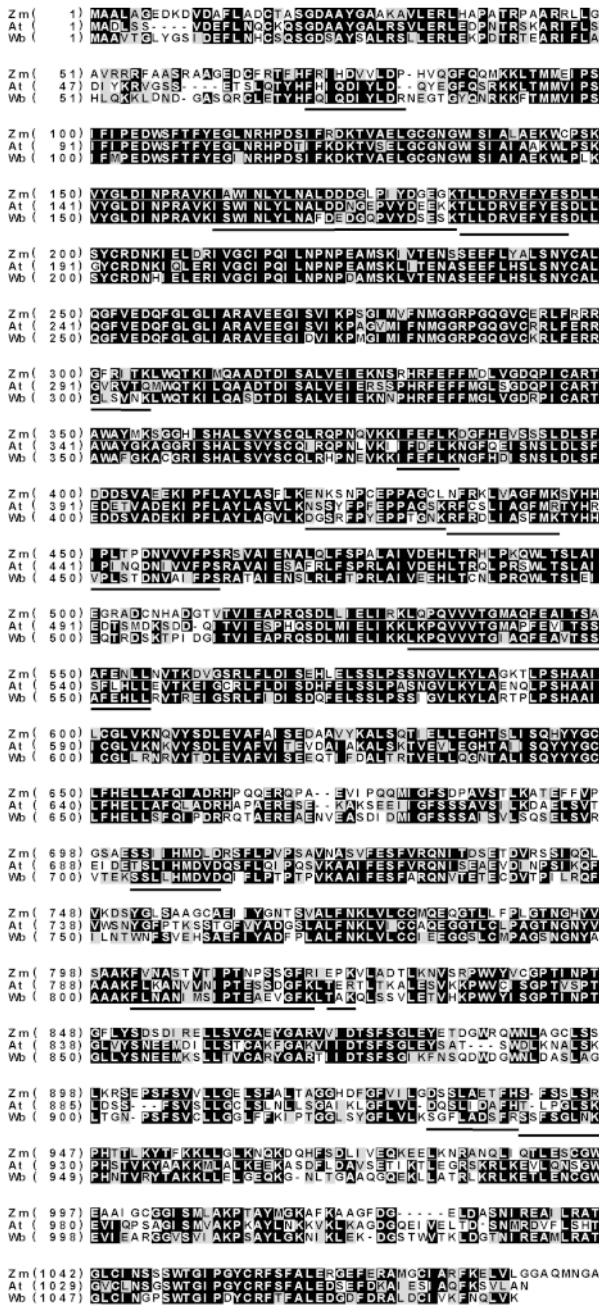


Figure 6. Deduced Amino Acid Sequences of *W. biflora*, Arabidopsis, and Maize MMTs.

The *W. biflora* sequence is a composite of the longest (3.1 kb) cDNA and a 5' RACE product; these overlapped by 517 bp and were identical in the overlap region. Peptide sequences from purified *W. biflora* MMT are underlined. Identical residues are shaded in black, similar residues in gray. GenBank accession numbers are AF 137023 for *W. biflora*, AF 144079 for maize, and AF 137380 for Arabidopsis. At, Arabidopsis; Wb, *W. biflora*; Zm, maize.

DISCUSSION

SMM and Sulfur Transport in the Phloem

Our data show that SMM makes a major contribution to the transport of reduced sulfur in the phloem in wheat and most probably in many other species. For wheat, the contribution of SMM is greater than that of GSH, which until now has been viewed as the major form in which reduced sulfur moves in the phloem (Rennenberg, 1982; Brunold and Rennenberg, 1997). SMM almost surely has been overlooked in the past for methodological reasons. For instance, several studies of phloem sap have used automated amino acid analyzer procedures in which SMM can coelute with other basic amino acids or NH₄⁺ (Skodak et al., 1965; Grunau and Swiader, 1991).

That SMM and GSH are abundant constituents of phloem sap (totaling ~3.5 mol% of α-amino nitrogen; Tables 1 and 2) may explain a “missing sulfur” anomaly noted in the nutrition of wheat grains (Fisher and Macnicol, 1986; Hayashi and Chino, 1986). The measured Met plus Cys plus SO₄²⁻ contents of wheat phloem sap, relative to total amino acids, are far too low to account for the ~4 mol% of Met plus Cys in grain proteins (Khan and Eggum, 1978). Most of the sulfur needed for grain protein synthesis must therefore arrive in some form other than Met, Cys, or SO₄²⁻. Our data indicate that this sulfur arrives as SMM and GSH.

SMM can be reconverted to Met via the action of HMT, which is present in seeds (Giovannelli et al., 1980). SMM arriving in the phloem at a level of ~2 mol% of amino acids, therefore, could readily provide the Met needed to synthesize grain proteins with an average Met content of ~2 mol%. There is good indirect evidence that SMM is a source of sulfur in seeds: it is essentially absent from mature grains and so must be metabolized (Wong et al., 1995; Pimenta et al., 1998), and conversion to Met is its only likely fate. Accordingly, we propose the scheme given in Figure 10, in which SMM is synthesized in leaves, moves to the grain, and is then recycled to Met for use in protein synthesis. This scheme does not posit a complete separation in space and time of the halves of the SMM cycle, because leaves express HMT (Mudd and Datko, 1990; Larina and Gessler, 1994) and seed tissues express MMT (Pimenta et al., 1998). Rather, it envisions that whereas both organs have the potential to perform the full cycle, flux is mainly from SMM in leaves and from SMM to Met in seeds. This implies that the SMM cycle is tightly regulated.

The same can be inferred by comparing the size of the flag leaf SMM pool (~85 nmol) to the flux of SMM out of this leaf. For the mid-grainfill period, this flux can be estimated by assuming that (1) the SMM content of phloem sap is 25 nmol mg⁻¹ dry weight (Table 1); (2) 1 mg of phloem sap is converted to 0.89 mg of grain dry matter (Penning de Vries et al., 1974; Fisher and Gifford, 1986); (3) ears have 40 grains, each growing at 2 mg dry weight per day (Fisher,

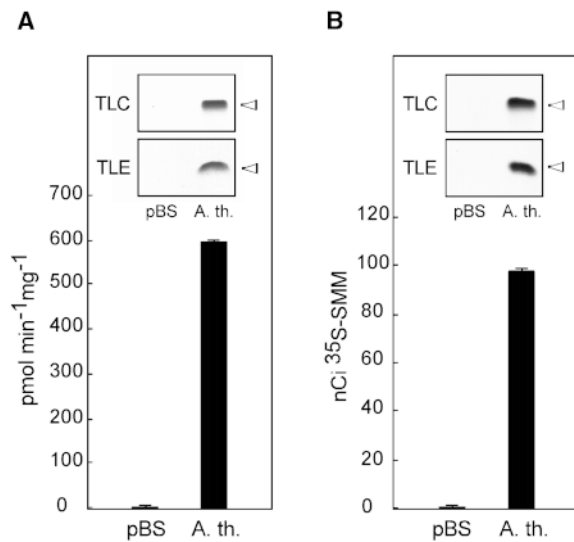


Figure 7. Functional Expression of Arabidopsis MMT in *E. coli*.

(A) MMT activity in *E. coli* strain DH10B harboring pBluescript (pBS) or pBS containing the full-length Arabidopsis MMT cDNA (A. th.). Data are means \pm SE of three observations. MMT was assayed radiometrically. Insets are autoradiographs of thin-layer chromatography (TLC) and thin-layer electrophoresis (TLE) analyses that confirm that the reaction product was ^{35}S -SMM; arrowheads mark the position of authentic SMM.

(B) Formation of ^{35}S -SMM from ^{35}S -Met by *E. coli* strain MTD123 harboring pBS or pBS containing the full-length Arabidopsis MMT cDNA. Cells were exposed to $9\ \mu\text{Ci}$ (18 nmol) of ^{35}S -Met for 65 to 75 min; they absorbed 3.7 to 4.4 μCi , of which 0.8 to 1.0 μCi remained in the soluble fraction. Data are means \pm SE of three observations. Insets are autoradiographs of TLC and TLE analyses that confirm the identity of the ^{35}S -SMM produced by cells expressing MMT. Arrowheads mark the position of authentic SMM.

1990); and (4) the flag leaf is the source of assimilates for the developing grains (Fisher, 1990). The leaf-grain SMM flux is therefore $(40 \times 2 \times 25/0.89) = 2250$ nmol per day. This daily flux is >20 -fold larger than the flag leaf SMM pool, which must therefore turn over approximately once per hour. Thus, wheat leaves do not stockpile SMM but produce it just before sending it to the grains—a strategy likely to require close metabolic control of the flux from Met to SMM.

Our finding that SMM is a prominent phloem constituent raises questions about the membrane transport processes implicated in its movement from leaves to sinks (Rentsch and Frommer, 1996). Assuming an apoplastic pathway of phloem loading, transmembrane movement must occur on release of SMM into the leaf apoplast, loading into the sieve element-companion cell complex and, in the grain, release from the maternal symplast into the endosperm cavity and uptake into the endosperm and embryo. Transport of amino acids into plant cells is known to involve a range of proton-coupled symporters with various substrate specificities,

some of which overlap (Rentsch et al., 1998). To date, SMM appears not to have been tested as a substrate for any of these. A priori, it could be a substrate for the known general amino acid or basic amino acid transporters (Chen and Bush, 1997; Rentsch et al., 1998); alternatively, there could be an SMM-specific transporter similar to the SMM permease of yeast (Rouillon et al., 1999). Identifying the transporter(s) responsible for the phloem loading of SMM is of particular interest, given that phloem loading could play an important role in determining the composition of the phloem sap and hence the amino acid content and nutritional value of seed storage proteins (Chen and Bush, 1997).

The Bipartite Structure of MMT and the Regulation of SMM Synthesis

An imperative for regulation might explain the unique bipartite structure of MMT in which a methyltransferase domain apparently is fused to a sequence related to the α family of PLP-dependent enzymes. Several considerations suggest that the PLP protein-like region is more likely to be a regulatory domain than a metabolic enzyme. First, it lacks a lysine in the usual position for the PLP binding site, having instead

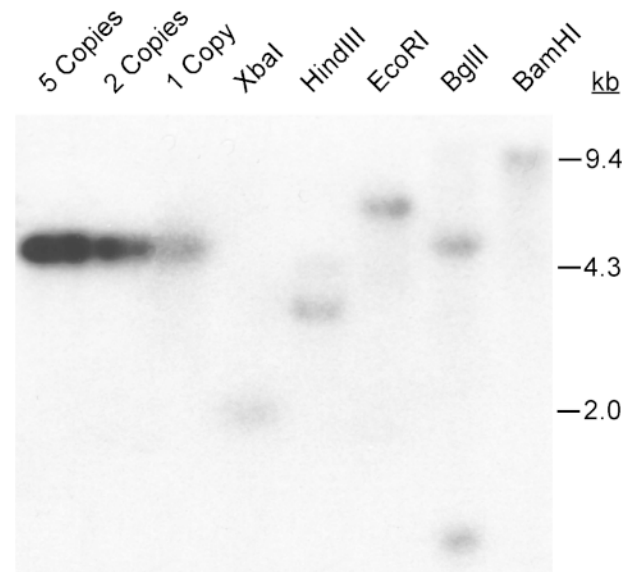
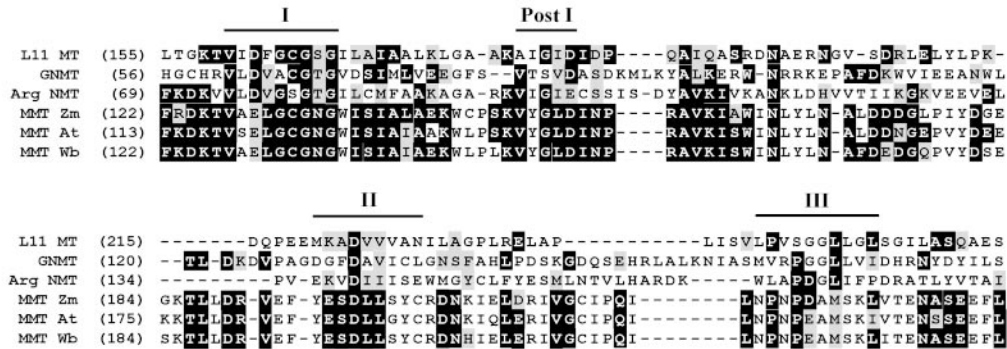


Figure 8. DNA Gel Blot Analysis of Arabidopsis MMT.

Arabidopsis genomic DNA (2.5 μg) was digested with the restriction enzymes indicated, blotted, and probed with a 1.2-kb MMT cDNA. Genomic reconstructions made with linearized plasmid DNA equivalent to one, two, or five copies per haploid genome were included. Hybridization and washing were at low stringency, as described in Methods. Hybridizing and washing at high stringency gave the same result. The positions of DNA length markers are shown at right.

A



B



C

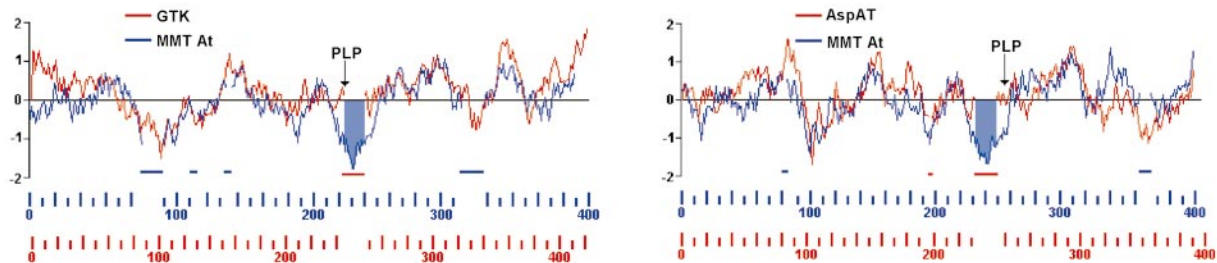


Figure 9. Evidence That MMTs Have a Methyltransferase Domain and a Domain Related to PLP-Dependent Proteins.

(A) Alignment of a region ~150 residues from the MMT N terminus with corresponding regions of three well-characterized methyltransferases (MT): *E. coli* ribosomal protein L11 MT (L11 MT; GenBank accession number 464465), rat glycine *N*-MT (GNMT; GenBank accession number 121328), and rat protein-arginine *N*-MT 1 (Arg NMT; GenBank accession number 2499804). Bars mark the conserved AdoMet binding motifs I, post-I, II, and III, as identified in L11 MT, GNMT, and Arg NMT (Kagan and Clarke, 1994; Gary et al., 1996). The consensus sequences for motifs I and post-I are hh(D/E)hGxGxG and hxxh(D/E), where h is a hydrophobic residue and x is any residue. Shading indicates residues that are identical (black) or similar (gray) in MMTs and the other enzymes. Dashes are gaps in the sequence to allow for maximal alignment. At, *Arabidopsis*; Wb, *W. biflora*; Zm, maize.

(B) Alignment of two regions ~250 and 40 residues from the C termini of MMTs, tomato 1-aminocyclopropane-carboxylate synthase (ACC syn; GenBank accession number 2118319), *Bacillus* sp aspartate aminotransferase (AspAT; GenBank accession number 112988), and rat glutamine transaminase K (GTK; GenBank accession number 1083706). Arrowheads mark residues (G-197, D/E-222, and R-386) found in all aminotransferases. Shading is as given in (A).

(C) Hydropathy profile alignments of the C-terminal region of a representative MMT (*Arabidopsis*, residues 663 or 673 to 1062) with GTK and AspAT. Profiles were predicted by the Kyte-Doolittle method, with a window length of 22 (GTK) or 19 (AspAT). Bars below the profiles indicate gaps introduced to maximize alignment. Arrows mark the position of the PLP binding lysine in the aminotransferases; that for GTK is as proposed by Abraham and Cooper (1996). Blue shading shows the hydrophobic region in MMT that apparently is inserted near the usual PLP binding site.

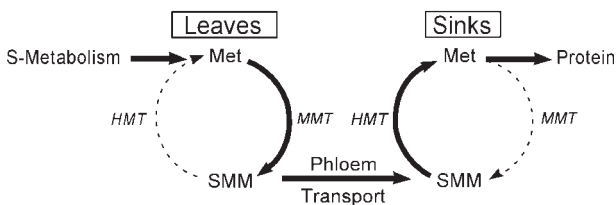


Figure 10. Possible Role of the SMM Cycle in Long-Distance Sulfur Transport.

A scheme showing a major flux (thick arrows) from Met to SMM in leaves, phloem transport of SMM, and reconversion of SMM to Met in developing seeds or other sinks. Dashed arrows indicate minor fluxes.

a stretch of hydrophobic residues. Second, its overall homology to α family enzymes is weak enough to make it unlikely to encode any known member of this group. Third, save for a hydrolysis not mediated by MMT (Gessler et al., 1991), SMM in most plants is metabolized only to Met, making it improbable that MMT has another catalytic activity against SMM. Finally, two other eukaryotic enzymes with pivotal positions in methyl group metabolism, glycine *N*-methyltransferase and methylenetetrahydrofolate reductase, are subject to metabolic regulation; in the reductase, the regulatory domain is a long C-terminal extension (Balaghi et al., 1993; Matthews et al., 1998). With respect to regulation, it may be significant that MMTs apparently exist in two forms that differ in gel mobility and activity or antigenicity (James et al., 1995a; Pimenta et al., 1998) and that barley MMT activity shows positive cooperativity for AdoMet (Lillo and Aarnes, 1980; Pimenta, 1996).

Supposing the C-terminal region to be regulatory, two observations suggest that it binds a ligand structurally related to Met. First, within the α family, MMTs are most similar to enzymes that act on Met or its analogs: glutamine transaminase K (which mediates Met synthesis from the corresponding α -keto acid), ACC synthase (which converts AdoMet to ACC), and aspartate aminotransferase (aspartate is the precursor of Hcy and Met). Second, the C-terminal region contains the conserved Arg-386 residue, which in aminotransferases interacts with the α -carboxyl group of the substrate (Jensen and Gu, 1996). Because *MMT* appears to be a single-copy gene in *Arabidopsis* and maize, it should be possible to probe the function of MMT and its domains by knocking out the native gene and subsequently introducing modified MMT sequences.

METHODS

Plant Materials

Wheat (*Triticum aestivum* cv SUN9E) was grown as described previously (Fisher, 1990) and used at the mid-grainfill stage for

phloem exudate collection via aphid stylets. *Wollastonia biflora* genotype H was grown as described previously (Trossat et al., 1996). Other plants (for exudate collection by the EDTA method, see below) were grown in a growth chamber (16-hr day, at 200 to 300 $\mu\text{mol m}^{-2} \text{sec}^{-1}$, at 22°C during the day and 18°C at night). These plants were *Arabidopsis thaliana* ecotype RLD, broad bean (*Vicia faba*), canola (*Brassica napus*), cucumber (*Cucumis sativus*), white lupine (*Lupinus albus*), maize (*Zea mays*), radish (*Raphanus sativus*), soybean (*Glycine max*), wheat (*T. aestivum* cv Bob White), and zucchini (*Cucurbita pepo*). All plants were used for experiments when they were at the flowering or seed-filling stage.

Collection of Phloem Exudates

Unlabeled or ^{35}S -labeled exudates were collected from the peduncles of wheat plants by using severed aphid stylets, as described by Fisher et al. (1992); other details are given in the text. For ^{35}S -labeling, the tip of the flag leaf was cut off and placed into 100 μL of solution containing 1 mCi of L - ^{35}S -Met (1000 Ci mmol^{-1} ; DuPont-New England Nuclear), which was taken up within 2 hr. One hour later, the terminal 2-cm section of the leaf was harvested; the rest of the blade was harvested at 72 hr. Wheat exudate samples were kept at -80°C until analysis. Labeled phloem sap was obtained from other species by EDTA-enhanced exudation (King and Zeevaart, 1974). A 5- μL droplet containing ^{35}S -Met (20 μCi) was applied to beveled tips of attached leaves, and the plants were left in the growth chamber for 2 hr. The labeled leaf was then severed, placed with its base in 1 mL of 5 mM Na_2EDTA , pH 7.0, and held in a humid chamber in darkness for 20 hr to permit exudation.

Analysis of ^{35}S -Labeled Compounds

Leaves were extracted as described previously (James et al., 1995b). Leaf extracts and phloem exudates were fractionated by passage through 1-mL columns of Dowex-1 (OH^-) and BioRex-70 (H^+ ; Bio-Rad) arranged in series; these columns retain, respectively, neutral plus acidic amino acids and *S*-methylmethionine (SMM; James et al., 1995b). Subsequent separations were by thin-layer chromatography (TLC) system 1 and thin-layer electrophoresis (TLE) system 2 (James et al., 1995b), or by TLC on cellulose plates developed with *n*-propanol-formic acid-water (20:1:5 [v/v]). Radioactivity was detected by autoradiography and amino acids with ninhydrin. The identity of ^{35}S -SMM was established by comigration with authentic SMM in the above systems, by decomposition upon treating with 1 M NaOH at 100°C for 2 hr (White, 1982), and by conversion to the α -hydroxy acid with nitrous acid (James et al., 1995b). For determination of configuration, ^{35}S -SMM (6 to 340 nCi) was treated for 16 hr at 24 to 25°C with 0.12 mL of a solution containing 63 mM potassium phosphate, pH 7.5, 2000 units of catalase, 2 μmol of D,L-SMM, and approximately equivalent activities (against SMM) of L- or D-amino acid oxidase (1 and 1.5 units, respectively). Reactions were run in 10×75 -mm glass tubes with Teflon-faced caps; the ^{35}S -dimethylsulfide (DMS) product was trapped in 10-mm filter discs containing 20 μL of 30% H_2O_2 and quantified by scintillation counting of the discs. Data were corrected for slight chemical decomposition of ^{35}S -SMM and for small differences in DMS yield from the L- and D-amino acid oxi-

dase reactions (measured by gas chromatography; Rhodes et al., 1997).

Analysis of SMM, Thiols, and Amino Acids

SMM analyses were conducted on BioRex-70 fractions. SMM in phloem samples was analyzed without derivatization by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) by using the instrumentation and procedures described by Trossat et al. (1998). SMM in leaf extracts was determined as described by Hanson et al. (1994). Thiols were analyzed by HPLC as their monobromobimane derivatives, as described by Herschbach et al. (1998); the γ -glutamylcysteinylserine peak was identified based on its chromatographic behavior (Klapheck et al., 1992) and quantified relative to a glutathione (GSH) standard. Amino acids were determined as described by Trossat et al. (1998).

cDNA Cloning and Sequence Analysis

S-Adenosylmethionine:Met S-methyltransferase (MMT) was purified to homogeneity from *W. biflora* leaves as described by James et al. (1995a); Figures 2 and 3 of James et al. (1995a) document the purity of the product. Peptides were obtained using endoproteinase Lys C or trypsin, separated by HPLC, and sequenced by Edman degradation or by mass spectrometry at the Michigan State and Harvard University facilities. mRNA was isolated from *W. biflora* leaves as described (Rathinasabapathi et al., 1997) and used to construct a cDNA library in the λ Uni-Zap XR vector (Stratagene, La Jolla, CA). The degenerate polymerase chain reaction (PCR) primers 5'-AARTTYTTRAAAYGCI AAYATHATG-3' (Y = T or C; R = A or G; H = T or C) and 5'-TTRAAICCIAC YTCRGCYTC-3' (corresponding to the ends of the peptide KFLNANIMSIPTAEVGFK) were used to amplify a 56-bp DNA fragment by using the cDNA library (2×10^8 plaque-forming units per 50- μ L reaction) as template. Sequencing confirmed that this fragment encoded the expected residues; a specific primer (5'-ATCATGTCTATCCCTACAGA-3') from its center was then used with the vector T7 primer to amplify the 1.1-kb 3' region of the MMT cDNA. The 1.1-kb fragment then was used to screen the library (3×10^4 plaques); this yielded >100 MMT cDNAs, all truncated, of which the longest was 3.1 kb. The 5' terminus was obtained by rapid amplification of cDNA ends (RACE), by using the Gibco BRL kit. Cloned RACE products from four independent reactions were sequenced; a clone with no errors (determined from the sequence consensus) was used to construct the complete cDNA by fusion at the NheI site.

Arabidopsis expressed sequence tag (EST) 205D23T7 (GenBank accession number H77211) was obtained from the Arabidopsis Biological Resource Center and shown to have a 1.2-kb insert encoding the C-terminal part of a protein homologous to *W. biflora* MMT. The insert then was used to isolate a full-length cDNA from an Arabidopsis (ecotype Landsberg *erecta*) leaf library in λ Uni-Zap XR (from T.L. Thomas, Texas A&M University, College Station). The maize EST was recovered from the collection at Pioneer Hi-Bred International Inc., Johnston, IA. Both strands of cDNAs were sequenced by using the ABI Prism dye terminator cycle sequencing Ready Reaction (PE Applied Biosystems, Warrington, UK) and an ABI model 373 sequencer. Sequence alignments were made using Multalin (Corpet, 1988) or Clustal W version 1.7 (Thompson et al., 1994) programs. Homology searches were made using BLAST programs (Altschul et al., 1997). Hydrophathy profile alignments were conducted via the Weizmann In-

stitute Bioinformatics server (<http://bioinformatics.weizmann.ac.il>), by using the Kyte-Doolittle calculation method.

Expression of MMT in *Escherichia coli*

For measurements of MMT activity in vitro, cultures of *E. coli* strain DH10B harboring the full-length Arabidopsis MMT cDNA or the empty pBluescript KS+ vector were grown to an OD_{600 nm} of 0.6 to 0.8 in of Luria-Bertani medium containing 100 μ g mL⁻¹ ampicillin and 1 mM isopropyl β -D-thiogalactopyranoside (IPTG). Soluble proteins were isolated by sonicating cells in Tris-Mes-acetate buffer, pH 7.2 (James et al., 1995a), containing 1 mM DTT and 10% (v/v) glycerol, and centrifuging for 15 min at 10,000g. MMT activity was assayed radiometrically (James et al., 1995a); protein concentrations were determined according to Bradford (1976). To confirm that Arabidopsis MMT is active in vivo in *E. coli*, we grew cultures of the strain MTD123 ($\Delta yagD \Delta metE \Delta meth$; Thanbichler et al., 1999) harboring the MMT cDNA or the pBluescript KS+ vector as above; cells from a 2-mL aliquot were then transferred to 2 mL of M9 medium containing 0.8% glucose, 1 mM IPTG, and 9 μ Ci (18 nmol) of ³⁵S-Met. When 40 to 50% of the ³⁵S-Met had been taken up (65 to 75 min), the cells were harvested and extracted in boiling water for 10 min. The soluble fraction was then analyzed for ³⁵S-SMM by ion exchange, TLC, and TLE as described above.

DNA Gel Blot Analysis

For Arabidopsis (ecotype RLD), genomic DNA was isolated from leaves as described (Dellaporta, 1994); 2.5 μ g of the isolated DNA was digested, separated in 1% agarose gels, and transferred to Duralon-UV membranes (Stratagene). Blots were both hybridized and washed at low or high stringency; the probe was the insert from EST 205D23T7. Low stringency was hybridization at 50°C and 1 M Na⁺, with a final wash at 22°C and 40 mM Na⁺; high stringency was hybridization at 65°C and 1 M Na⁺, with a final wash at 65°C and 20 mM Na⁺ (Taylor et al., 1993). For maize (genotype Hi-II), genomic DNA was prepared from leaves as described (Richards, 1997); 6 μ g of digested DNA was separated as above and blotted to Hybond-N+ membranes (Amersham). Blots were hybridized at 60 or 65°C (Sambrook et al., 1989) with a 0.5-kb maize MMT genomic fragment comprising part of the region between the 5' EcoRV and PstI sites, and washed at low or high stringency (final wash in 0.1 \times SSC [1 \times SSC is 0.15 M NaCl and 0.015 M sodium citrate] and 0.1% SDS at 22 or 65°C, respectively). Probes were labeled with phosphorus-32 by a random hexamer priming method.

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S-Methylmethionine Plays a Major Role in Phloem Sulfur Transport and Is Synthesized by a Novel Type of Methyltransferase

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