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Methionine Metabolism in Plants

CHLOROPLASTS ARE AUTONOMOUS FOR *DE NOVO* METHIONINE SYNTHESIS AND CAN IMPORT *S*-ADENOSYLMETHIONINE FROM THE CYTOSOL*

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The subcellular distribution of Met and *S*-adenosylmethionine (AdoMet) metabolism in plant cells discloses a complex partition between the cytosol and the organelles. In the present work we show that *Arabidopsis* contains three functional isoforms of vitamin B_{12}-independent methionine synthase (MS), the enzyme that catalyzes the methylation of homocysteine to Met with 5-methyltetrahydrofolate as methyl group donor. One MS isoform is present in chloroplasts and is most likely required to methylate homocysteine that is synthesized *de novo* in this compartment. Thus, chloroplasts are autonomous and are the unique site for *de novo* Met synthesis in plant cells. The additional MS isoforms are present in the cytosol and are most probably involved in the regeneration of Met from homocysteine produced in the course of the activated methyl cycle. Although Met synthesis can occur in chloroplasts, there is no evidence that AdoMet is synthesized anywhere but the cytosol. In accordance with this proposal, we show that AdoMet is transported into chloroplasts by a carrier-mediated facilitated diffusion process. This carrier is able to catalyze the uniport uptake of AdoMet into chloroplasts as well as the exchange between cytosolic AdoMet and chloroplastic AdoMet or *S*-adenosylhomocysteine. The obvious function for the carrier is to sustain methylation reactions and other AdoMet-dependent functions in chloroplasts and probably to remove *S*-adenosylhomocysteine generated in the stroma by methyltransferase activities. Therefore, the chloroplastic AdoMet carrier serves as a link between cytosolic and chloroplastic one-carbon metabolism.

The sulfur-containing amino acid Met is essential in all organisms as a building block of proteins and as a component of the universal activated methyl donor *S*-adenosylmethionine (AdoMet).[1] By using the aquatic plant *Lemna pausicostata*, Giovanelli *et al*. (1) have shown that the synthesis of AdoMet accounts for ~80% of Met metabolism, whereas the synthesis of proteins (the only pathway consuming the entire Met molecule) drives ~20% of Met. More than 90% of AdoMet is then used for transmethylation, leading to nucleic acid, protein, lipid, and other metabolite modifications (1). Utilization of the methyl group of AdoMet in transmethylation is accompanied by recycling of the homocysteinyl moiety and regeneration of Met, a set of reactions designated as the activated methyl cycle.

Most of the genes and enzymes involved in Met and AdoMet synthesis and metabolism have been characterized in plants (for reviews see Ref. 2–4). One of the most intriguing findings is the complex subcellular distribution of these metabolic routes. The first two reactions specific for *de novo* Met synthesis consist of the conversion of cysteine into homocysteine (Hcy) by the enzymes cystathionine γ-synthase and cystathionine β-lyase. Both enzymes are present only in the chloroplasts (2). Hcy is then methylated to Met by transfer of the methyl group of 5-methyltetrahydrofolate (5-CH_3-H_4PteGlu$_n$), a reaction catalyzed by methionine synthase (MS). There are two types of MS, a cobalamin-dependent enzyme that contains a vitamin B_{12} cofactor and a cobalamin-independent isoenzyme (5). To date, only the cobalamin-independent MS activity has been described in higher plants (6–9), although cobalamin-dependent enzymes also occur in the photosynthetic protist *Euglena gracilis* (10). Plant MS seems to be present only in the cytosol (6–9), thus implying that Hcy originating from cysteine has to exit the chloroplast to be converted to Met.

MS not only catalyzes the last reaction in *de novo* Met synthesis but also serves to regenerate the methyl group of AdoMet after methylation reactions. In plants as in other eukaryotes, the two other enzymes participating in the activated methyl cycle, namely AdoMet synthetase and *S*-adenosylhomocysteine (AdoHcy) hydrolase, are most probably present exclusively in the cytosol (2, 3, 11). As a consequence, it is usually considered that chloroplasts and mitochondria must import AdoMet from the cytosol, principally to fulfill methylation reactions. Also, because AdoHcy produced during methylation reactions is a potent competitive inhibitor of methyltransferases, it is assumed that chloroplasts and mitochondria have to export AdoHcy to the cytosol to maintain the AdoMet/AdoHcy ratio that regulates methyltransferase activities (for a review see Ref. 12). In support of these statements, mitochondria isolated from rat liver were found to transport AdoMet from the cytosol via a carrier-mediated system that is inhibited by AdoHcy (13). Recently, mitochondrial carriers capable of

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ608673, AJ608674, and AJ608675.

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[1] The abbreviations used are: AdoMet, *S*-adenosylmethionine; Hcy, homocysteine; 5-CH_3-H_4PteGlu$_n$, 5-methyltetrahydrofolate; MS, methionine synthase; AdoHcy, *S*-adenosylhomocysteine; chl, chlorophyll; GFP, green fluorescent protein; PMSF, phenylmethylsulfonyl fluoride; MOPS, 4-morpholinepropanesulfonic acid; RT, reverse transcriptase.

TABLE I
Synthetic oligonucleotides used in this study

Primers	Sequences (5′ → 3′)
cDNA cloning	
MS1–5′	GAGAATTCTCAAAATGGCTTCACACATTGT
MS1–3′	GAGACTGCAGCCTTGTTCAAATCAAGCTTTTC
MS2–5′	GAGAATTCCAAAAATGGCTTCCCACATTG
MS2–3′	GAGACTGCAGTATAGAAATAATATCTTCAAGCTC
MS3–5′	AGACCCGGGAATGGGTCAGCTCGCTCTTC
MS3–3′	GAGACTGCAGGAGGAAACTTACTTGAGGAAAG
pET23d cloning	
MS1-pET5′	GAGATCATGAGTTCACACATTGTTGGATACCC
MS1-pET3′	GAGAGAATTCTTCACTTGGCACTGGCG
MS2-pET5′	GAGATCATGAGTTCCCACATTGTTGGATATCC
MS2-pET3′	GAGAGAATTCTTCACTTGGCACTACCGAG
MS3-pETM49	GAGATCATGAGTTCCCACATCGTTGGGTATCC
MS3-pET3′	GAGACTCGAGTTTATGACTTATTTAGCTGAGAGCG
p35Ω-sGFP(S65T) cloning	
MS1GFP5′	GAGAGAGTCGACAATGGCTTCACACATTGTTGG
MS1GFP3′	GAGAGATCATGAGTTCAGCATAGGCACCAGTA
MS2GFP5′	GAGAGAGTCGACAAATGGCTTCCCACATTGTTG
MS2GFP3′	GAGAGATCATGAGCTCAGCATAGGCACCGC
MS3GFP5′	GAGAGAGTCGACAATGGGTCAGCTCGCTCTTC
MS3GFP3′	GAGAGATCATGAGAGAGTACGCATCGGAAAATG
Real time PCR	
MS1-A	CTGTGGTCTCAAGACCCGT
MS1-B	CCTTGTTCAAATCAAGCTTTTC
MS2-A	ACTGTGGTCTGAAGACAAGG
MS2-B	TATAGAAATAATATCTTCAAGCTC
MS3-C	CTCAGAAAATCCGAGCAAAAG
MS3-D	CCTTCATGGAAGACTGATAAG
Actin-5′	GTTTTGCTGGGGATGATGCACC
Actin-3′	CGTTTGGATTGAGCTTCATCGCC

exchanging cytosolic AdoMet for mitochondrial AdoHcy were cloned and characterized in yeast (14) and human (15). Such a transport system has never been described for plant mitochondria or plastids.

The aim of the present work was to explore the role of chloroplasts in relation to Met and AdoMet metabolism. First, we have shown the existence of a cobalamin-independent MS in the plastids, thus rendering this compartment autonomous for *de novo* Met synthesis from its precursors cysteine, aspartate, and 5-CH_3-H_4PteGlu$_n$. In *Arabidopsis*, two additional isoforms of B_{12}-independent MS are present in the cytosol and are most probably devoted to the recycling of Met in the course of the activated methyl cycle. Second, we found that AdoMet is transported from the cytosol to chloroplasts by a carrier-mediated facilitated diffusion process. Besides the uniport uptake of AdoMet, the carrier can catalyze the exchange between cytosolic AdoMet and chloroplastic AdoMet or AdoHcy, thus suggesting that it can be involved in the prevention of AdoHcy accumulation in the stroma.

EXPERIMENTAL PROCEDURES

Plant Material—Arabidopsis thaliana (ecotype Wassilewskija) plants were grown in soil under greenhouse conditions (23 °C with a 16-h photoperiod and a light intensity of 200 μmol of photons m^{-2} s^{-1}) until harvested for analysis. *A. thaliana* (ecotype Columbia) cell suspension cultures were grown under continuous white light (40 μmol of photons m^{-2} s^{-1}) at 23 °C with rotary agitation at 125 rpm in Gamborg's B5 medium supplemented with 1 μM 2-naphthalene acetic acid and 1.5% (w/v) sucrose. Pea (*Pisum sativum* L. var. Douce Provence) plants were grown for 9 days under a 12-h photoperiod (140 μmol of photons m^{-2} s^{-1}) at 22 °C (day) and 20 °C (night). Etiolated pea plants were grown in complete darkness at 20 °C, and leaves were collected under a green safelight. Freshly harvested spinach (*Spinacia oleracea* L.) leaves were obtained from a local grower and stored at 4 °C until required for use.

Chemicals—(*6R,6S*)-5-Methyl-5,6,7,8-tetrahydropteroyl-L-glutamatic acid (5-CH_3-H_4PteGlu$_1$) and L-homocysteine thiolactone were obtained from Sigma. Pteroylpenta-γ-glutamic acid was purchased from Schircks Laboratories (Jona, Switzerland). [*carboxyl*-^{14}C]AdoMet (2.18 GBq $mmol^{-1}$ in 0.2 M H_2SO_4, Amersham Biosciences) was mixed with AdoMet (*p*-toluene sulfonate salt, Sigma) and neutralized with $BaCO_3$ as described by Teyssier *et al.* (16).

Cloning of AtMS1–3 cDNAs—Poly(A)$^+$ mRNAs were isolated from above-ground parts of 3-week old *A. thaliana* plants and used to construct a Marathon™ cDNA amplification library (17). Full-length cDNAs were generated by PCR using primers overlapping the region containing the initiation Met codon and the 3′-untranslated region of each gene (Table I). Amplification was done with the *Pfu* DNA polymerase according to the manufacturer's instructions (Promega). PCR products were subcloned into the pBluescriptII KS vector digested with SmaI and sequenced (GenomeExpress, Meylan, France). The cloned sequences were designated AtMS1, -2, and -3 for *A. thaliana* methionine synthase homologs.

Expression of AtMS Proteins in Escherichia coli—The coding sequences of the AtMS cDNAs were amplified from pBluescript plasmid templates by high fidelity PCR using *Pfu* DNA polymerase and primers listed in Table I. For overexpression of AtMS1 and AtMS2, the full-length coding sequences were amplified, digested with BspHI and EcoRI, and inserted between the NcoI and EcoRI sites of pET-23d (Novagen) to give plasmids pET-MS1 and pET-MS2. To express AtMS3 in *E. coli*, the targeting region was removed so that the initiation codon of the recombinant protein was Met-49. The resulting truncated cDNA was digested with BspHI and XhoI and cloned between the NcoI and XhoI sites of pET-23d to give plasmid pET-MS3. The pET-MS constructs were introduced first into *E. coli* DH5α and then into BL21(DE3)pLysS cells (Stratagene). Transformed cells were grown at 37 °C in LB medium containing carbenicillin (100 μg ml^{-1}), chloramphenicol (34 μg ml^{-1}), and 0.5 mM $ZnSO_4$ until A_{600} reached 0.6. Isopropylthio-β-D-galactoside was added (final concentration 0.5 mM), and the cells were further grown for 4 h at 28 °C (AtMS1), 16 h at 28 °C (AtMS2), or 16 h at 16 °C (AtMS3). Subsequent operations were done at 4 °C. Cells were pelleted (4,000 × *g*, 20 min), resuspended in 50 mM Tris-HCl, pH 7.5, 10 mM β-mercaptoethanol, 5% (v/v) glycerol, 1 mM PMSF, and a mixture of protease inhibitors (catalog number 1873580, Roche Applied Science), and disrupted by sonication (Sonifier 250, Branson). The soluble protein extracts were separated from the cell debris by centrifugation at 16,000 × *g* for 15 min and desalted on PD-10 columns (Amersham Biosciences) equilibrated in 50 mM Tris-HCl, pH 7.5, 10 mM β-mercaptoethanol, and 5% (v/v) glycerol.

Measurement of Cobalamin-independent MS Activity—Cobalamin-independent MS was assayed using unlabeled 5-CH_3-H_4PteGlu$_n$ as a substrate, the reaction product tetrahydrofolate being detected spectrophotometrically following its conversion to 5,10-methenyltetrahydrofo-

late by heating with formic acid (18). The standard assays contained, in a final volume of 400 μl, 10 mM potassium phosphate buffer, pH 7.2, 50 mM Tris-HCl, pH 7.2, 0.1 mM $MgSO_4$, 10 mM dithiothreitol, 2 mM Hcy, 150 μM (*6R,6S*)-5-CH_3-H_4PteGlu$_5$ or (*6R,6S*)-5-CH_3-H_4PteGlu$_1$, and desalted protein extracts. The reaction was initiated by the addition of Hcy and incubated at 30 °C for 4–30 min. The reaction was stopped by adding 100 μl of 5 N HCl, 60% (v/v) formic acid and heating for 14 min at 84 °C. After equilibration at room temperature, the assays were centrifuged at 16,000 × *g* for 5 min to remove precipitated proteins. The absorbance of the supernatant was measured at 350 nm, and 5,10-methenyltetrahydrofolate was quantified using an absorption coefficient of 26,500 M^{-1} cm^{-1} (18). The L-Hcy stock solution was prepared by hydrolysis of the L-Hcy thiolactone, and the actual concentration was determined by titration with 5,5′-dithiobis-2-nitrobenzoic acid (18). (*6R,6S*)-5-CH_3-H_4PteGlu$_5$ was prepared from pteroylpenta-γ-glutamic acid as described by Yeo and Wagner (19). 5-CH_3-H_4PteGlu$_n$ substrates were prepared as 5 mM solutions in 10 mM sodium ascorbate and manipulated under a stream of argon in order to protect them from oxidation.

Isolation of Chloroplasts, Mitochondria, and a Cytosolic-enriched Fraction from Arabidopsis—Chloroplasts from *A. thaliana* leaves were purified on Percoll™ (Amersham Biosciences) gradients as described by Awai *et al.* (20). Intact chloroplasts were lysed in 10 mM MOPS, pH 7.6, 4 mM $MgCl_2$, 1 mM PMSF, 1 mM aminocaproic acid. Chloroplast subfractions were separated on a step gradient of 0.93 to 0.6 M sucrose in 10 mM MOPS, pH 7.6, by centrifugation at 70,000 × *g* for 1 h. The soluble fraction (stroma) was collected at the top of the 0.6 M sucrose layer. Mitochondria were prepared from 6-day-old *Arabidopsis* cell suspension cultures using the procedure described by Ravanel *et al.* (17). Crude mitochondria were layered on discontinuous gradients consisting of 18, 23, and 40% Percoll layers in 0.3 M sucrose, 10 mM potassium phosphate, pH 7.2, 1 mM EDTA, 0.1% (w/v) bovine serum albumin. Gradients were centrifuged at 39,000 × *g* for 40 min and mitochondria collected at the 23:40% Percoll interface. In order to obtain soluble proteins (matrix), purified mitochondria were lysed by a 10-fold dilution in 10 mM MOPS, pH 7.2, 1 mM dithiothreitol, 1 mM PMSF, 1 mM aminocaproic acid and submitted to three freeze-thaw cycles. Membranes were removed by centrifugation at 100,000 × *g* for 1 h. Cytosolic enriched fractions were prepared from *Arabidopsis* protoplasts obtained by enzymatic digestion of 6-day-old cell suspension cultures using the procedure described by Ravanel *et al.* (17). Briefly, protoplasts were gently ruptured by passing through a 20-μm nylon mesh and subsequently through a 10-μm nylon mesh. The protoplast lysate was centrifuged successively at 100 × *g* for 5 min, 900 × *g* for 5 min, and 13,000 × *g* for 20 min. The 13,000 × *g* supernatant fraction, which is enriched in the cytosolic marker activity and contains a small part of mitochondrial and chloroplastic marker enzymes (17), was further centrifuged at 100,000 × *g* for 1 h to remove membranes.

Isolation of Plastids and Mitochondria from Pea Leaves—For the preparation of plastids and mitochondria, green or etiolated leaves were collected from 9-day-old seedlings. Mitochondria from green leaves were isolated and purified as described by Douce *et al.* (21) by using two steps of purification on Percoll/polyvinylpyrrolidone gradient. Mitochondria from etiolated leaves were isolated using the procedure described for potato tuber mitochondria with one step of purification on a Percoll gradient (21). Chloroplasts were isolated and purified on a continuous Percoll gradient as described by Douce and Joyard (22). Etioplasts were purified from etiolated pea leaves as described by Jabrin *et al.* (23) by using a preformed 30–80% (v/v) Percoll density gradient. Soluble proteins were obtained from the purified pea organelles after osmotic lysis and centrifugation, as described for the *Arabidopsis* subcellular fractions.

Antibody Production and Immunoblot Analysis—The AtMS1 protein fused to maltose-binding protein was purified by affinity chromatography and injected into guinea pigs to raise antibodies (7). Total soluble proteins from *Arabidopsis* and pea leaves were extracted by grinding powdered samples in 50 mM Tris-HCl, pH 7.5, 10 mM β-mercaptoethanol, 5% (v/v) glycerol, 1 mM PMSF, and 1 mM aminocaproic acid. Samples were centrifuged at 16,000 × *g* for 20 min at 4 °C, and the supernatant was used as a source of soluble proteins. Proteins were measured by the method of Bradford (24) using bovine serum albumin as standard. Proteins from *Arabidopsis* or pea fractions were resolved by SDS-PAGE and electroblotted to nitrocellulose membrane. The blots were probed using the AtMS1 antibodies (dilution 1:3000), horseradish peroxidase-conjugated anti-guinea pig IgGs, and detection was achieved by chemiluminescence.

Transient Expression of GFP Fusion Proteins in Arabidopsis Protoplasts—The GFP reporter plasmid 35Ω-sGFP(S65T) expressing an engineered version of the green fluorescent protein under the control of the cauliflower mosaic virus 35S promoter (25) was used for transient expression experiments in *Arabidopsis* protoplasts. The N-terminal regions of the AtMS1, -2, and -3 proteins fused upstream and in-frame with sGFP(S65T) contained 229, 229 and 275 residues, respectively. These regions were PCR-amplified using *Pfu* DNA polymerase and specific flanking primers listed in Table I. The PCR products were digested with SalI and BspHI and inserted between the SalI and NcoI sites of p35Ω-sGFP(S65T) to give plasmids pGFP-MS1, pGFP-MS2, and pGFP-MS3. *Arabidopsis* protoplasts prepared from a 4-day-old cell suspension culture were transformed essentially as described by Abel and Theologis (26). For each transformation 300 μl of protoplasts (~2 × 10^6 cells) were mixed with 20–30 μg of plasmid DNA, 50 μg of salmon sperm carrier DNA, and 350 μl of polyethylene glycol solution (0.4 M mannitol, 0.1 M $CaNO_3$, 40% (w/v) PEG-4000), and incubated at room temperature for 30 min. Protoplasts were cultured in 3 ml of medium (0.4 M sucrose, Murashige and Skoog basal medium, 4 mM $CaCl_2$, and 250 mg l^{-1} xylose) under dim light at 22 °C for up to 48 h. Samples were analyzed by fluorescence microscopy using a Zeiss Axioplan2 fluorescence microscope, and the images were captured with a digital charge-coupled devices camera (Hamamatsu). The filter sets used were Zeiss filter set 13, 488013-0000 (exciter BP 470/20, beamsplitter FT 493, emitter BP 505–530, and Zeiss filter set 15, 488015-0000 (exciter BP 546/12, beamsplitter FT 580, emitter LP 590) for GFP and chlorophyll fluorescence, respectively.

Real Time PCR—Relative quantification of *atms1*, *atms2*, and *atms3* mRNAs was done by real time PCR using Lightcycler System (Roche Applied Science) and Lightcycler-Faststart DNA Master SYBR Green I kit. Poly(A) mRNAs from different organs (leaves, stems, roots, flowers, siliques, and seeds) of *Arabidopsis* were prepared using Straight A's™ mRNA Isolation System according to the manufacturer's instructions (Novagen). First strand cDNA was synthesized from 1 μg of mRNA in a final volume of 20 μl using oligo$(dT)_{20}$ primers (Thermoscript RT-PCR System, Invitrogen). For each real time PCR experiment, 5 μl of DNA preparation was used as a template in a standard 10-μl LightCycler PCR with appropriate primers (Table I) used at a final concentration of 1 μM and 3 mM $MgCl_2$. Amplification and detection were performed using the following profile: 95 °C for 8 min followed by 40 cycles of 95 °C for 10 s, 66 °C for 15 s, and 72 °C for 8 s. Data were analyzed with the LightCycler Relative Quantification software (Roche Applied Science) using the amplification of actin cDNA (GenBank™ accession number U39449, primers listed in Table I) as internal standard of mRNA integrity and cDNA preparation. The specificity of the reaction was verified by melting curve analysis obtained by increasing temperature from 55 to 95 °C (0.1 °C/s).

Measurement of AdoMet Uptake by Intact Chloroplasts—Uptake of AdoMet was measured by silicone oil-filtering centrifugation (27) using spinach leaf chloroplasts purified according to Mourioux and Douce (28). The intactness of the chloroplasts was tested by using an oxygen electrode and was found to be close to 95%. Chlorophyll (chl) content was determined as described by Bruinsma (29). Polypropylene tubes (0.4 ml) were filled with 20 μl of 1 M $HClO_4$, 70 μl of AR200 silicone oil (Wacker-Chemie GmbH, Munich, Germany), and 140 μl of medium A (0.33 M sorbitol, 50 mM Hepes-KOH, pH 7.2) containing various concentrations of the radiolabeled substrate. Uptake was started by addition of 10 μl of freshly prepared chloroplasts (80–100 μg of chl/assay) using a small plastic spatula to achieve rapid dispersion of the chloroplasts in the medium. After incubation for 10–500 s at 25 °C under low light conditions, uptake was stopped by centrifuging the tubes at 3,000 × *g* for 2 min (swinging bucket, Kubota KM-15200 microcentrifuge). An aliquot of the supernatant was taken for determination of the substrate-specific radioactivity, and a 10-μl aliquot of the $HClO_4$ phase was taken for determination of radioactivity incorporated into chloroplasts. The kinetics of AdoMet uptake were fitted to a single exponential by using Equation 1, where A_t and A_{eq} are the amount of AdoMet incorporated in the chloroplasts at time *t* and at the equilibrium, and k_{obs} is the pseudo-first order rate constant.

$$A_t = A_{eq}(1 - \exp(-k_{obs}t)) \quad \text{(Eq. 1)}$$

To analyze AdoMet/AdoHcy counter-exchanges across the chloroplast envelope, freshly prepared chloroplasts were loaded with AdoMet or AdoHcy by incubation in the dark with 100 μM unlabeled substrates for 10 min at room temperature. The chloroplasts were quickly cooled down on ice for 10 min and then washed twice by dilution with ice-cold medium A and centrifugation at 2,500 × *g* for 3 min. The pelleted chloroplasts were gently resuspended in medium A and analyzed for their ability to import 100 μM [*carboxyl*-^{14}C]AdoMet, as described

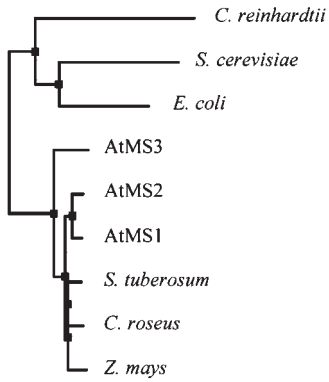

FIG. 1. **Similarity tree based on multiple sequence alignment of B_{12}-independent methionine synthases.** The tree was produced from a multiple sequence alignment (MultAlin version 5.4.1; Ref. 30) including B_{12}-independent methionine synthases from *Arabidopsis* (this work), *C. roseus* (GenBank™ accession number CAA58474), *Zea mays* (GenBank™ accession number AAL33589), *Solanum tuberosum* (GenBank™ accession number AAF74983), *E. coli* (GenBank™ accession number AAC76832), *S. cerevisiae* (GenBank™ accession number AAB60301), and *C. reinhardtii* (GenBank™ accession number AAC49178).

above. For back-exchange experiments, chloroplasts were loaded with 100 μM [*carboxyl*-^{14}C]AdoMet for 10 min at 25 °C, cooled down on ice for 10 min, and then washed twice in ice-cold medium A. Back-exchange was measured by incubating 10 μl of [*carboxyl*-^{14}C]AdoMet-loaded chloroplasts in 140 μl of medium A containing either unlabeled AdoHcy or AdoMet (each at 100 μM). The efflux of [*carboxyl*-^{14}C]AdoMet was followed by silicone oil-filtering centrifugation as described above.

Measurement of AdoMet and AdoHcy—Intact chloroplasts (50–70 mg chl) were lysed in 2 ml of distilled water and centrifuged for 20 min at 16,000 × *g* and 4 °C to remove membrane systems. AdoMet and AdoHcy present in the supernatant were separated from other nucleotides by ion-paired high pressure liquid chromatography using a reversed phase UP3HDO-15 M column (3-μm particle size; Interchim) and quantified using a photodiode array detector (Waters). AdoMet and AdoHcy were identified by co-elution with authentic standards and diode array analysis of UV spectra. The A_{256} was used for quantification.

RESULTS

Arabidopsis Contains Three Isoforms of B_{12}-independent MS—The first goal of our study was to identify and characterize all the genes that may encode MS in *Arabidopsis*, in particular regarding the occurrence of an Hcy-methylating enzyme in the chloroplast. A search in the *Arabidopsis* Information Resource data base (www.arabidopsis.org) revealed three genes coding putative cobalamin-independent MS in the *Arabidopsis* genome (*Arabidopsis* Genome Initiative entries At5g17920, At3g03780, and At5g20980). The full-length cDNAs were obtained by RT-PCR and designated AtMS1, AtMS2, and AtMS3, respectively. The AtMS1 and AtMS2 cDNAs encode 765-residue (~84 kDa) polypeptides that are 92% identical to each other and 87–89% identical to other known B_{12}-independent MS from higher plants. The deduced AtMS3 protein (812 residues, ~90 kDa) shares 79–81% identity with AtMS1, AtMS2, and other known plant MS. A summary of the primary sequences alignment using a similarity tree (Fig. 1) clearly indicates that AtMS3 is apart from other plant cobalamin-independent MS proteins identified so far. When compared with B_{12}-independent MS from other organisms, the AtMS proteins are 49% identical to MetE from *E. coli*, 47% identical to Met6p from *Saccharomyces cerevisiae*, and 41% identical to B_{12}-independent MS from *Chlamydomonas reinhardtii* (Fig. 1).

To prove that the AtMS clones encode functional B_{12}-independent MS enzymes, the three *Arabidopsis* cDNAs were subcloned into the expression vector pET-23d and introduced into the *E. coli* strain BL21(DE3)pLysS. Overexpression of AtMS1 and AtMS2 led to soluble gene products comprising up to 30% of the total cellular proteins. AtMS3 was expressed in *E. coli* as a truncated protein with an N-terminal 48-residue deletion. The expressed protein was not an abundant protein in crude extracts, but its presence in the soluble fraction was ascertained by Western blot experiments (data not shown). Enzyme activity was determined using the non-radioactive assay described by Drummond *et al.* (18) with (*6R,6S*)-5-CH_3-H_4PteGlu$_5$ as a substrate. By using our standard incubation conditions, the bacterial endogenous MetE activity was negligible compared with the activity measured when the plant proteins were expressed. As shown in Table II, the three *Arabidopsis* proteins are functional B_{12}-independent MS capable of methylating Hcy to Met with 5-CH_3-H_4PteGlu$_5$ as methyl donor. The ~40-fold difference in activities between AtMS1 and AtMS2 on the one hand and AtMS3 on the other hand is because of the relative abundance of the proteins in the *E. coli* crude extracts. The apparent K_m values for 5-CH_3-H_4PteGlu$_5$ were ~60 μM for the AtMS1 and AtMS2 enzymes, and 17 μM for AtMS3 (Table II). The substrate specificity of the *Arabidopsis* B_{12}-independent MS enzymes was further analyzed using the monoglutamate form of 5-CH_3-H_4PteGlu$_n$ as methyl donor. As shown in Table II, 5-CH_3-H_4PteGlu$_1$ cannot replace efficiently the polyglutamate substrate, a ratio of at least 200:1 in favor of the pentaglutamate being observed for AtMS1 and AtMS2. These properties are consistent with previous reports (8, 31–32) showing that the cobalamin-independent MS from *E. coli*, yeast, or higher plants are unable to accept 5-CH_3-H_4PteGlu$_1$ as methyl donor.

MS Isoforms Are Present in the Cytosol and the Chloroplasts—AtMS3 is the only B_{12}-independent MS described in higher plants that is predicted to possess a targeting sequence. Indeed, analysis of the N-terminal extension of AtMS3 using the prediction softwares TargetP (33) and Predotar (version 0.5; www.inra.fr/predotar/) revealed features typical for mitochondrial or chloroplastic transit peptides, thus suggesting that this protein is targeted to the organelles. To verify this prediction, we first analyzed the subcellular distribution of B_{12}-independent MS isoforms in *Arabidopsis* by Western blot. A preliminary experiment indicated that antibodies rose against AtMS1 cross-reacted with both AtMS2 and AtMS3 recombinant proteins (data not shown). As shown in Fig. 2, total soluble extracts from *Arabidopsis* or pea leaves contain two polypeptides of 84 ± 1 and 81 ± 1 kDa, respectively, which react with the antibodies. The occurrence of these polypeptides was then analyzed in purified chloroplasts and mitochondria and in a cytosolic enriched fraction (see "Experimental Procedures"). Fig. 2 clearly indicates that the high molecular weight MS polypeptide is detected in the cytosolic enriched fraction but not in the organellar fractions. In *Arabidopsis*, the low molecular weight MS polypeptide was detectable in soluble proteins obtained from purified chloroplasts (stroma) but was absent in purified mitochondria (matrix). This immunological pattern was confirmed with organelles purified from pea plants developed in light or in the dark (Fig. 2; the faint band detected in mitochondria from etiolated leaves is attributable to a cross-contamination by plastids). Thus, these results indicate that B_{12}-independent MS is present in both the cytosol and the stromal space of plastids (chloroplasts and etioplasts).

To analyze the subcellular localization of the AtMS proteins *in vivo*, the N-terminal regions (approximately one-third of the proteins) were fused upstream to the GFP marker protein. As shown in Fig. 3, the expression of AtMS1-GFP and AtMS2-GFP in *Arabidopsis* protoplasts resulted in green fluorescence throughout the cytoplasm and the nucleus, a pattern similar to the one observed with GFP alone. These results demonstrate

TABLE II

Kinetic properties of B_{12}-independent MS isoforms from Arabidopsis

Activity measurements were made at 30 °C using desalted crude extracts obtained from *E. coli* BL21(DE3)pLysS cells expressing the AtMS1, AtMS2, or AtMS3 cDNAs. For activity determinations, the 5-CH_3-H_4PteGlu$_n$ substrate was used at a final concentration of 150 μM. For the determination of the K_m values for 5-CH_3-H_4PteGlu$_5$, the Hcy concentration was set at 2 mM. Data are means of three replicates ± S.D. ND, not detected.

	AtMS1	AtMS2	AtMS3
Activity with 5-CH_3-H_4PteGlu$_5$ (nmol min^{-1} mg^{-1} protein)	26.5 ± 3.7	20.7 ± 1.5	0.6 ± 0.1
Activity with 5-CH_3-H_4PteGlu$_1$ (nmol min^{-1} mg^{-1} protein)	≤0.1	≤0.1	ND
K_m for 5-CH_3-H_4PteGlu$_5$ (μM)	60 ± 7	57 ± 6	17 ± 3

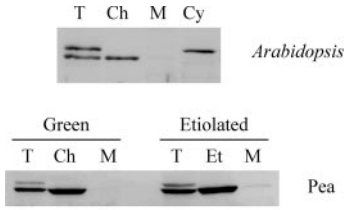

FIG. 2. **Western blot analysis of MS isoforms in subcellular fractions from *Arabidopsis* and pea.** Total soluble extracts, purified plastids and mitochondria, and cytosolic-enriched fractions were obtained from *Arabidopsis* or pea plants as described under "Experimental Procedures." Proteins were separated by SDS-PAGE using 10% acrylamide gels, transferred to nitrocellulose membranes, and probed with the antibodies raised against AtMS1. The amounts of proteins loaded on the gels are 50 μg for the total extracts (*T*), 30 μg for the chloroplast (*Ch*) or etioplast (*Et*) stroma, 20 μg for mitochondrial matrix (*M*), and 20 μg for the cytosolic enriched fraction (*Cy*). The faint immunolabeling of the low molecular weight MS isoform in the mitochondrial extract from etiolated pea leaves is because of a cross-contamination of this fraction by etioplasts (10–15% as judged by carotenoid measurements).

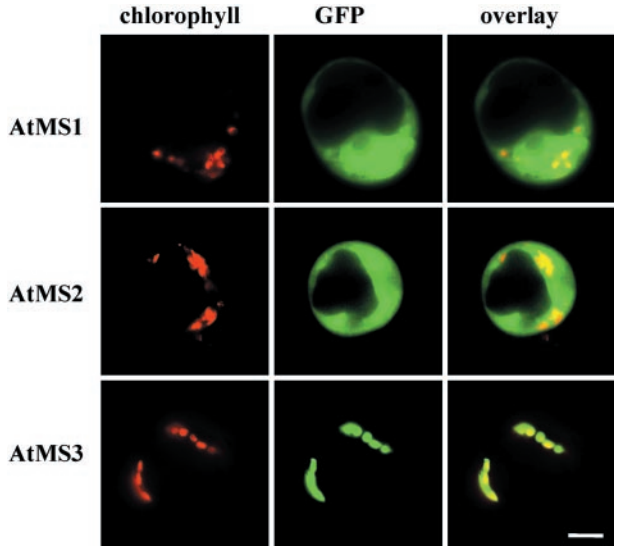

FIG. 3. **Expression of GFP fused to the N-terminal regions of the AtMS proteins in *Arabidopsis* protoplasts.** Constructs encoding fusion proteins between the N terminus of AtMS isoforms and the engineered reporter protein sGFP(S65T) were introduced into *Arabidopsis* protoplasts as described under "Experimental Procedures." GFP (*green pseudo-color*) and chlorophyll (*red pseudo-color*) fluorescence was observed by fluorescence microscopy using a Zeiss Axioplan2 microscope. *Scale bar*, 10 μm.

that AtMS1 and AtMS2 are cytosolic proteins. Expression of the AtMS3-GFP fusion protein in protoplasts resulted in a pattern of green fluorescence that colocalized with the red autofluorescence of chlorophyll (Fig. 3). These data indicate that AtMS3 contains a functional plastid targeting sequence, a result in good agreement with the Western blot analysis shown in Fig. 2. It should be noted that a small part of protoplasts transformed with AtMS3-GFP displayed green fluorescence in chloroplasts and in punctate structures scattered throughout the cytosol (data not shown). These small particles were identified as mitochondria. The physiological significance of this last finding is not known because Western blots presented in Fig. 2 clearly indicate that mitochondria do not contain a B_{12}-independent MS isoform. Also, the AtMS3-GFP fusion protein is under the control of the cauliflower mosaic virus 35S promoter and thus very likely expressed at a high non-physiological level in protoplasts, a situation that may disturb the sorting machinery.

Cytosolic and Chloroplastic MS Expression Patterns—To have insight into the physiological function of the three MS isoforms, we examined the expression of the *atms* genes in different organs from *Arabidopsis* by quantitative real time RT-PCR. As shown in Fig. 4*A*, the *atms1*, *atms2*, and *atms3* mRNAs were detected in all analyzed organs (roots, stems, leaves, flowers, siliques, and seeds), demonstrating that the corresponding genes were transcriptionally active. In the growth conditions used, the three genes displayed, however, considerably different steady-state levels and expression profiles. Indeed, the *atms1* mRNA coding the first cytosolic MS isoform was the most abundant transcript in all organs, with the highest level in flowers and the lowest level in roots. The *atms2* mRNA coding the second cytosolic MS isoform largely followed the *atms1* mRNA expression pattern and was generally 4–10-fold less abundant than *atms1*, with the exception of flowers where the ratio is 80:1 in favor of *atms1* (Fig. 4*A*). The *atms3* gene displayed a fairly constant and very low expression level in stems, leaves, flowers, and siliques. In these organs the mRNA coding the plastidial MS isoform is 65–430-fold less abundant than the mRNAs coding the cytosolic isoforms. The ratio is less marked in roots and in dry seeds where the steady-state level of *atms3* mRNA is 2.4-fold lower than *atms1* and 4-fold higher than *atms2* (Fig. 4*A*).

To analyze the correlation between mRNA and protein abundance, Western blots were realized using soluble protein extracts obtained from the different organs. As mentioned above the 81-kDa polypeptide detected by Western blot corresponds to the chloroplastic AtMS3 protein, whereas the 84-kDa band most probably corresponds to the two cytosolic proteins that are not distinguishable by size. To make sure that both AtMS1 and AtMS2 are present in the high molecular weight band, we separated a soluble protein extract from *Arabidopsis* cells by two-dimensional gel electrophoresis, identified the cytosolic protein(s) by Western blot, and analyzed the protein spot of interest by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectroscopy. The obtained peptide mass map indicated that both AtMS1 and AtMS2 proteins are present in the protein spot, thus indicating that the immunolabeling of the 84-kDa band is representative of the two cytosolic MS isoforms. Comparison of the mRNA and protein expression patterns in Fig. 4, *A* and *B,* indicated that there is no direct relationship between the accumulation of MS isoforms and the transcription level of the *atms* genes. Indeed, roots contain the lowest level of mRNAs coding AtMS1 and AtMS2 but display a large amount of the cytosolic enzymes. On the contrary, dry seeds and more particularly flowers contain high

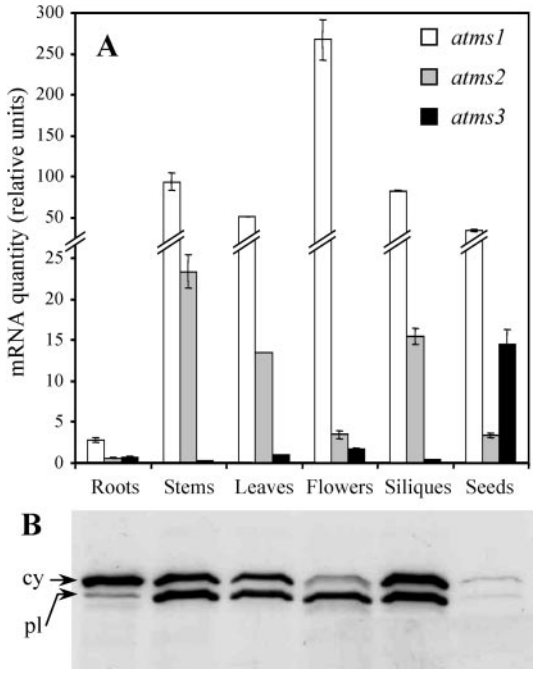

FIG. 4. **Expression profiles of the *atms* mRNAs and proteins in *Arabidopsis* organs.** *A*, steady-state levels of *atms* mRNAs were measured by real time RT-PCR on poly(A)$^+$ RNA from *Arabidopsis* organs using isoform-specific primers (Table I). Data are means of three independent experiments performed with four cDNA dilutions ± S.D. The amplification of actin cDNA has been used as internal standard of RNA integrity and cDNA preparation. *B*, total soluble proteins (50 μg) from *Arabidopsis* organs were separated by SDS-PAGE using 10% acrylamide gels, transferred to nitrocellulose membranes, and probed with the antibodies raised against AtMS1. The high and low molecular weight bands correspond to the cytosolic (*cy*) and plastidial (*pl*) MS isoforms, respectively.

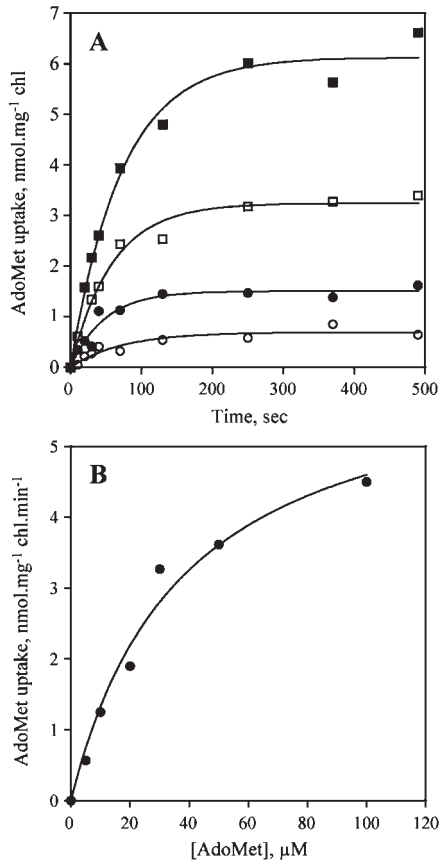

FIG. 5. **Time and concentration dependence of the uptake of AdoMet into spinach chloroplasts.** *A*, chloroplasts were incubated with various concentrations of [*carboxyl*-^{14}C]AdoMet for the times indicated at 25 °C, and reactions were stopped by silicone oil-filtering centrifugation. The [*carboxyl*-^{14}C]AdoMet concentrations used are 5 μM (○), 20 μM (●), 50 μM (□), and 100 μM (■). Curves were fitted to a single exponential as described under "Experimental Procedures." *B*, initial rates of AdoMet uptake as a function of external [*carboxyl*-^{14}C]AdoMet concentrations. The curve was fitted with the Michaelis-Menten equation thus giving K_m and V_{max} values of 38 μM and 6.3 nmol mg^{-1} chl min^{-1} at 25 °C, respectively.

levels of the *atms1* and *atms2* transcripts, but the corresponding proteins do not accumulate. A similar variation between mRNA and protein levels is also observed for AtMS3. With the exception of seeds, the mRNA coding the chloroplastic MS is present at very low levels in all organs, but the immunolabeling of the protein is as strong as the one for the cytosolic isoforms (Fig. 4). The opposite situation is observed in dry seeds where the *atms3* mRNA is at least 10 times more abundant than in other organs, but the protein is barely detectable. To summarize, these data indicate that the accumulation of MS isoforms in *Arabidopsis* organs is not only determined by the steady-state level of the corresponding mRNAs but also by post-transcriptional events.

Chloroplasts Import AdoMet by a Facilitated Diffusion Process—Although the above-mentioned results indicate that Met synthesis can occur in chloroplasts, there is no evidence that AdoMet is synthesized anywhere but the cytosol. This observation along with the presence of AdoMet-dependent reactions in chloroplasts leads to the proposal that chloroplasts have to import AdoMet from the cytosol. To test this hypothesis, we analyzed AdoMet uptake by intact spinach leaf chloroplasts using silicone oil-filtering centrifugation. Fig. 5*A* shows the uptake kinetics obtained for various concentrations of [*carboxyl*-^{14}C]AdoMet. This figure indicates that AdoMet was taken up very rapidly by chloroplasts and leveled off after 2–4 min under our experimental conditions. Fig. 5*A* also indicates that the final concentration of AdoMet reached in the stroma at the equilibrium is proportional to the concentration of AdoMet initially present in the incubation medium. We ascertained that the radioactive product incorporated into chloroplasts was AdoMet by co-migration with authentic [*carboxyl*-^{14}C]AdoMet using thin layer chromatography (data not shown). This analysis also indicated that under our experimental conditions [*carboxyl*-^{14}C]AdoMet was not used for methylation reactions in the chloroplast because [*carboxyl*-^{14}C]AdoHcy could not be detected. A plot of the initial rate of AdoMet uptake *versus* substrate concentration in the medium shows a saturable curve, which is indicative of a carrier-mediated transport (Fig. 5*B*). From these data the calculated apparent K_m and V_{max} values were 38 μM and 6.3 nmol mg^{-1} chl min^{-1}, respectively. To investigate whether the transport of AdoMet across the chloroplast envelope is energy-dependent, uptake experiments were performed either in light or in darkness, or with or without ATP-Mg. These treatments had no effect on the kinetic parameters of AdoMet uptake (results not shown), thus indicating that ATP hydrolysis or light-induced transmembrane proton potential across the thylakoid membranes was not involved. Thus the uptake of AdoMet into chloroplasts is mediated by a facilitated diffusion process.

AdoMet Uptake into Chloroplasts Is Inhibited by AdoHcy—The possibility that transport of AdoMet is mediated by the chloroplastic adenylate carrier, an antiport that exchanges cytosolic for stromal adenylic nucleotides, was ruled out because ATP (up to 1 mM) had no effect on the uptake of AdoMet. The addition of either Met or Hcy (up to 100 μM) to the external medium did not lead to any modification of the kinetic parameters of the AdoMet carrier (results not shown). Our results

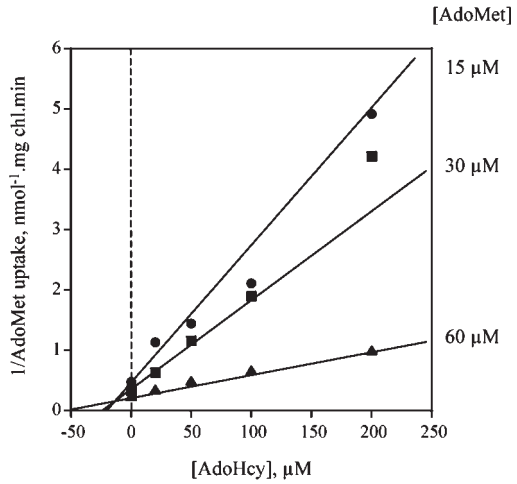

FIG. 6. **Inhibition of AdoMet uptake into chloroplasts by AdoHcy.** Kinetics of [*carboxyl*-^{14}C]AdoMet uptake into chloroplasts were done with various concentrations of radioactive AdoMet and unlabeled AdoHcy. The initial rates of AdoMet uptake were measured and plotted against AdoHcy concentration for the three AdoMet concentrations indicated. The K_i value for AdoHcy calculated from this Dixon plot is 14 μM.

argue therefore against the proposal that AdoMet is transported by putative Met or Hcy carriers. On the other hand, the AdoHcy molecule that is formed during the course of AdoMet-dependent methylation reactions was found to be a potent inhibitor of AdoMet uptake into chloroplasts. The uptake of AdoMet was measured in the presence of various concentrations of AdoHcy, and the Dixon plot presented in Fig. 6 shows that the inhibition is competitive. The apparent K_i value calculated from the Dixon plot was 14 μM. This result indicates that AdoHcy, in contrast with ATP, Met, and Hcy, is well recognized by the AdoMet-binding site of the carrier.

AdoMet/AdoHcy Counter-exchanges Take Place across the Chloroplast Envelope—The possibility that transport of AdoMet across the chloroplast envelope is mediated by a carrier acting as an antiport that exchanges external AdoMet (or AdoHcy) for internal AdoHcy (or AdoMet) was tested by two complementary experiments. First, chloroplasts were loaded with 100 μM [*carboxyl*-^{14}C]AdoMet for 10 min at 25 °C, cooled down at 4 °C, rapidly and extensively washed, and incubated at 25 °C into exchange medium containing either unlabeled AdoHcy or AdoMet (each at 100 μM). The kinetics of [*carboxyl*-^{14}C]AdoMet efflux shown in Fig. 7*A* indicate that radiolabeled AdoMet is released from chloroplasts in the absence of external AdoMet or AdoHcy with an initial rate of ~2 nmol mg^{-1} chl min^{-1} at 25 °C. This result indicates that the AdoMet carrier is able to catalyze a unidirectional transport (uniport). Also, Fig. 7*A* shows that the addition of AdoMet or AdoHcy to the exchange medium caused an ~8-fold increase of the efflux of the chloroplastic [*carboxyl*-^{14}C]AdoMet as compared with the medium without substrate. These results indicate that exchanges between internal AdoMet and external AdoMet or AdoHcy had occurred.

Second, we measured the uptake of [*carboxyl*-^{14}C]AdoMet into chloroplasts pre-loaded with unlabeled AdoMet or AdoHcy. We have verified on separate experiments that the pre-loading of chloroplasts with 100 μM unlabeled AdoMet led to a significant increase in the stromal concentration of AdoMet from ~15 μM up to 120 ± 25 μM (mean ± S.D., n = 4). Also, the pre-loading of chloroplasts with 100 μM AdoHcy led to an increase in the stromal concentration of AdoHcy from an undetectable value up to 90 ± 22 μM (mean ± S.D., n = 5). The variations observed in the final concentrations of substrates in the stroma were attributable to the unidirectional transport of AdoMet or AdoHcy that occurred during the course of chloroplast washing (the efflux is considerably reduced when this procedure is carried out at low temperature). The pre-loading of chloroplasts with either AdoMet or AdoHcy led to a marked stimulation of [*carboxyl*-^{14}C]AdoMet uptake into the chloroplasts (Fig. 7*B*). Under standard conditions (25 °C) the influx is so rapid that it was difficult to measure accurately the initial rate of [*carboxyl*-^{14}C]AdoMet uptake into pre-loaded chloroplasts. Decreasing the temperature from 25 to 4 °C led to a marked decrease in the maximum rate of AdoMet uptake so that we could determine that chloroplasts pre-loaded with AdoMet and AdoHcy took up external AdoMet ~10 and ~6 times more rapidly than untreated chloroplasts, respectively. These results indicate that external AdoMet is taken up by chloroplasts in counter-exchange with AdoMet or AdoHcy.

DISCUSSION

The subcellular compartmentation of the final step of Met synthesis in plants is still a matter of questioning and debate more than 3 decades after the first report of MS activity in plant extracts (34). This enzyme catalyzes a unique reaction, the methylation of Hcy to Met with 5-CH_3-H_4PteGlu$_n$ as methyl group donor, but is required for two functions, namely to ensure *de novo* synthesis of Met from Hcy produced in chloroplasts and to regenerate the methyl group of AdoMet following transmethylation reactions. It has been speculated that this dual function may be catalyzed by MS isoenzymes with distinct subcellular locations and possibly distinct catalytic mechanisms, *i.e.* vitamin B_{12}-dependent or -independent activities (2). A survey of the *Arabidopsis* genome indicated that there is no plant homolog of the B_{12}-dependent MS found in bacteria, mammals, or the photosynthetic protozoa *E. gracilis*. *Arabidopsis* contains, however, three functional isoforms of the B_{12}-independent enzyme, one of which is located in plastids (Figs. 3 and 4). This result suggests strongly that this isoform is devoted to *de novo* synthesis of Met and that these organelles are autonomous for the synthesis of Met (Fig. 8). The two other MS isoforms in *Arabidopsis* are located in the cytosol and most probably are dedicated to the regeneration of Met, because this compartment contains the two other key enzymes of the activated methyl cycle, namely AdoMet synthetase and AdoHcy hydrolase (Fig. 8).

The plastid localization of AtMS3 is consistent with the fact that the chloroplast is the site of Hcy synthesis through the trans-sulfuration pathway (2) and that this organelle contains a pool of 5-CH_3-H_4PteGlu$_n$ (36), the only fate of this compound is the synthesis of Met. This compartmentation is also in accordance with the subcellular location of the enzyme folylpolyglutamate synthetase that is involved in the synthesis of the glutamate tail of tetrahydrofolate coenzymes (17). The presence of this enzyme in plastids is of crucial importance because the chloroplastic MS, similarly to the cytosolic isoforms, cannot efficiently utilize the monoglutamate form of 5-CH_3-H_4PteGlu$_n$ as a methyl donor for Hcy methylation (Table II). The facts that 5-CH_3-H_4PteGlu$_1$ was routinely used to measure MS activity in plant extracts and that this substrate replaces laboriously the polyglutamate form of 5-CH_3-H_4PteGlu$_n$ are probably the reasons why chloroplastic MS activity has never been detected (see for example Ref. 37). Finally, the presence of MS in plastids is supported by the observation that isolated chloroplasts incubated with either [^{14}C]aspartate or [^{35}S]SO_4^{2-} can synthesize radiolabeled Met (38, 39).

Both the *de novo* synthesis of Met in plastids and its recycling in the cytosol are ubiquitous reactions in the plant cell. Indeed, the different MS isoforms could be detected by Western blot in all the *Arabidopsis* organs examined, namely roots,

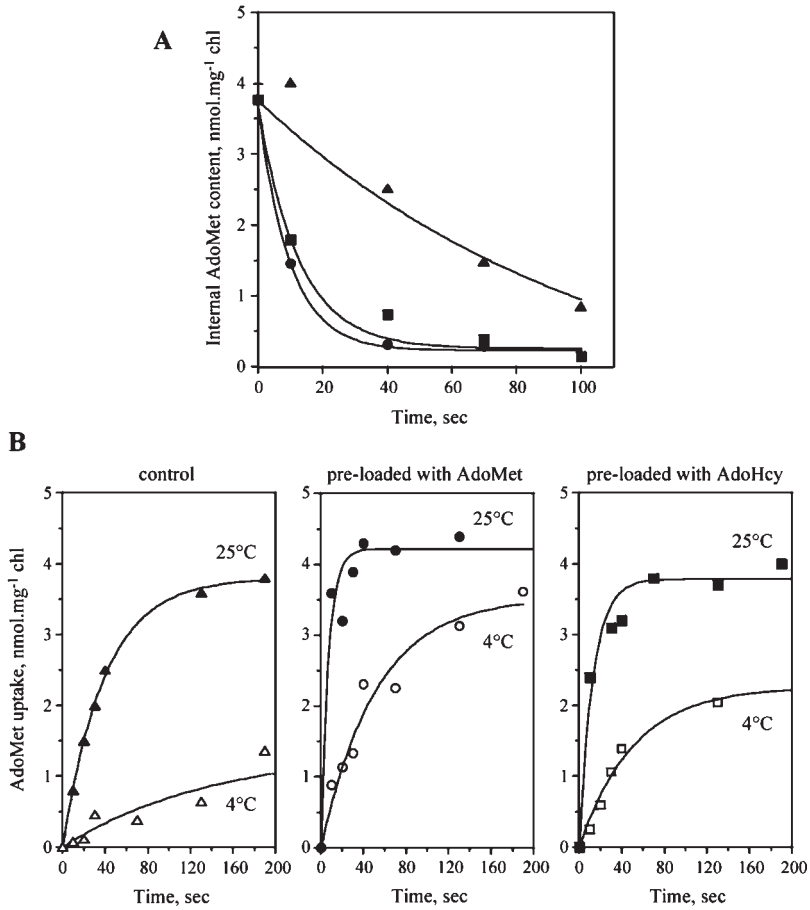

FIG. 7. **Effects of internal and external AdoMet and AdoHcy concentrations on the transport of [*carboxyl*-^{14}C]AdoMet across the chloroplast envelope.** *A*, kinetics of [*carboxyl*-^{14}C]AdoMet efflux from chloroplasts incubated with external unlabeled AdoMet or AdoHcy. Chloroplasts were loaded with 100 μM [*carboxyl*-^{14}C]AdoMet, washed with ice-cold medium, and used for back-exchange experiments in medium containing 100 μM unlabeled AdoMet (●), 100 μM unlabeled AdoHcy (■), or no addition (▲). *B*, kinetics of [*carboxyl*-^{14}C]-AdoMet uptake into chloroplasts pre-loaded with unlabeled AdoMet or AdoHcy. Chloroplasts were loaded with 100 μM unlabeled AdoMet or AdoHcy, washed with ice-cold medium, and analyzed for their ability to import 100 μM [*carboxyl*-^{14}C]AdoMet at 25 or 4 °C. In control experiments, chloroplasts were similarly treated in medium containing neither AdoMet nor AdoHcy. Curves were fitted to a single exponential as described under "Experimental Procedures."

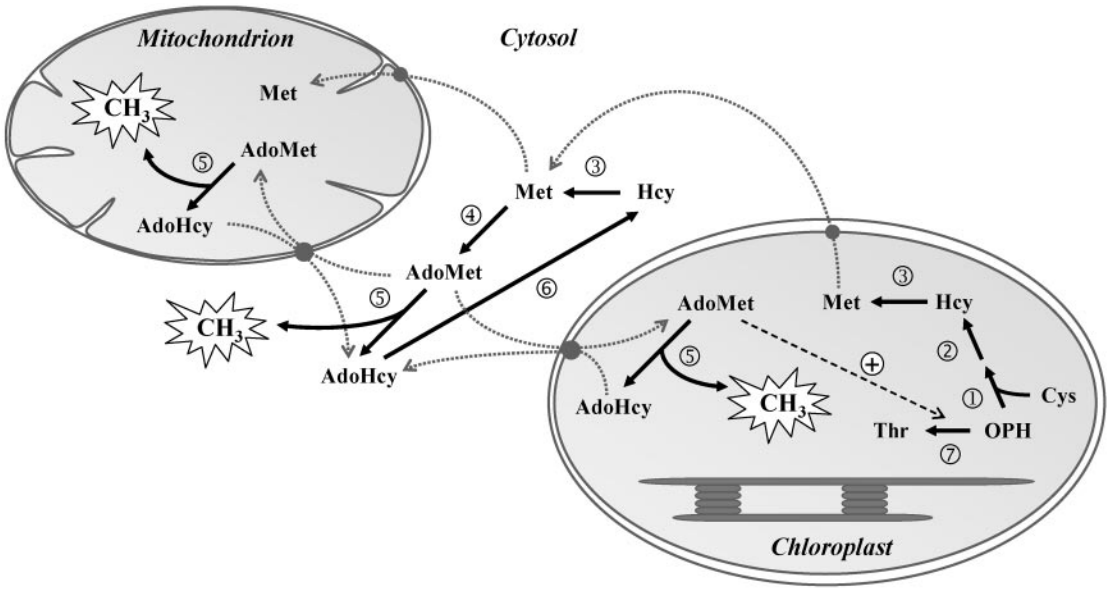

FIG. 8. **Working model of the subcellular compartmentation of Met and AdoMet metabolism in plant cells.** The enzymes involved in Met and AdoMet metabolism are as follows: *1*, cystathionine γ-synthase; *2*, cystathionine β-lyase; *3*, methionine synthase; *4*, AdoMet synthetase; *5*, AdoMet-dependent methyltransferases; *6*, AdoHcy hydrolase. The reaction catalyzed by threonine synthase (*7*) is allosterically activated by AdoMet (35). The chloroplastic AdoMet/AdoHcy carrier, its putative mitochondrial homolog, and the putative Met carriers are indicated by *circles*. *OPH*, *O*-phosphohomoserine.

stems, leaves, flowers, siliques, and seeds. Also, the plastidial and cytosolic proteins could be identified in both green and etiolated pea leaves, thus indicating that their accumulation is independent on light. As noticed previously in *Catharanthus roseus* cell cultures (8) and potato plants (9, 40), we found that there is not always a good correlation between the level of the mRNAs coding the three MS isoforms in *Arabidopsis* and the abundance of the corresponding proteins (Fig. 4). Thus, the steady-state mRNA level for one isoform can vary up to 2 orders of magnitude between two organs with only moderate alteration of the protein level. Similarly, Nikiforova *et al.* (40) reported that the significant reduction in MS mRNA levels in potato plants submitted to a drought stress was not accompanied by modifications of the protein level. Taken together, these

data indicate that the accumulation of MS polypeptides in the cytosol and chloroplasts is tightly controlled at both the transcriptional and post-transcriptional levels in order to maintain the activities of *de novo* Met synthesis and the activated methyl cycle. Another example of the tight control of Met synthesis and recycling concerns the germination process. A proteomic analysis of *Arabidopsis* seed germination indicated that the cytosolic AtMS1 isoform was present at low levels in dry mature seeds, and its level increased 4-fold after 1 day of imbibition (41). We found that the cytosolic and chloroplastic MS isoforms are present at low level in dry seeds (Fig. 4*B*) and that the amount of both proteins increased in 1-day imbibed seeds (data not shown). It is conceivable that the *atms1* and *atms3* mRNAs that accumulated to high levels in dry seeds (Fig. 4*A*) are rapidly translated during the imbibition process to allow an accumulation of both the cytosolic and chloroplastic enzymes. This regulatory mechanism is consistent with the observation that both the *de novo* Met biosynthetic pathway and the activated methyl cycle must be switched on very early during seed germination (41).

In plants as in other eukaryotes, the synthesis of AdoMet is localized exclusively in the cytosol thus indicating that this compound must be imported into the organelles where it is required for methylation reactions and other metabolic functions (Fig. 8). The transport of AdoMet across the mitochondrial inner membrane was first demonstrated by Horne *et al.* (13) using rat liver mitochondria. More recently, the mitochondrial carrier for AdoMet was identified and characterized in yeast (14) and in human (15). In the present work we have shown that AdoMet is transported across the chloroplast envelope by a saturable carrier. Because the concentration of AdoMet was almost negligible in our chloroplast preparations and because the import of AdoMet operates independently of energy, this transport system is referred to as facilitated diffusion. Further properties of AdoMet transport suggested that chloroplastic AdoMet carrier is able to catalyze the uniport uptake of AdoMet into chloroplasts as well as the exchange between cytosolic AdoMet and chloroplastic AdoMet or AdoHcy. In the absence of specific inhibitors it is difficult to discriminate the situation where a single carrier or two distinct carriers (one acting as a uniport and the other acting as an antiport) are involved. However, the kinetic properties we observed here for the chloroplastic AdoMet carrier are analogous to those of the yeast mitochondria AdoMet carrier (Sam5p protein) reconstituted into liposomes (14), thus suggesting the involvement of a single chloroplastic carrier. For these two transport systems the rate of the exchange reaction is ~10 times higher than that of the uniport uptake.

The obvious physiological function for the transport of AdoMet into the chloroplast is principally to sustain various methylation reactions. For example, it can be stated that the syntheses of chlorophyll (42), tocopherols, and plastoquinones (43) require key methylation steps and that several proteins of the thylakoid membranes and the stroma, including the subunits of ribulose-bisphosphate carboxylase/oxygenase, are methylated (44). The other role of the AdoMet carrier is probably to remove AdoHcy generated in the stroma during methylation reactions, first to prevent its accumulation and second to facilitate its integration in the cytosolic activated methyl cycle. The first rationale is suggested by the commonly accepted statement that the AdoMet/AdoHcy ratio determines the activity of methyltransferases (12). However, it must be stated that the AdoMet/AdoHcy ratio in chloroplasts is probably high (15 μM AdoMet *versus* undetectable levels of AdoHcy, see "Results") and that few chloroplastic methyltransferases have been characterized in sufficient detail to make meaningful comparisons regarding their sensitivity to changes in the AdoMet/AdoHcy ratio. The second rationale is suggested by the presence of all the enzymatic machinery necessary for a rapid regeneration of AdoMet from AdoHcy in the cytosol (Fig. 8). To conclude, it must be mentioned that another role of AdoMet in chloroplasts is to activate threonine synthase in an allosteric manner and thus to modulate the partition of the *O*-phosphohomoserine flux between the Met and Thr biosynthesis pathways (Fig. 8; Ref. 35). Therefore, the chloroplastic AdoMet carrier serves as a link between cytosolic and chloroplastic one-carbon metabolism and as a key element in the regulation of the synthesis of the aspartate-derived amino acids in the chloroplast.

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REFERENCES

1. Giovanelli, J., Mudd, S. H., and Datko, A. H. (1985) *Plant Physiol.* **78,** 555–560
2. Ravanel, S., Gakière, B., Job, D., and Douce, R. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 7805–7812
3. Hanson, A. D., and Roje, S. (2001) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **52,** 119–137
4. Hesse, H., and Hoefgen, R. (2003) *Trends Plant Sci.* **8,** 259–262
5. Gonzalez, J. C., Banerjee, R. V., Huang, S., Sumner, J. S., and Matthews, R. G. (1992) *Biochemistry* **31,** 6045–6056
6. Eichel, J., Gonzalez, J. C., Hotze, M., Matthews, R. G., and Schroder, J. (1995) *Eur. J. Biochem.* **230,** 1053–1058
7. Gakière, B., Job, D., Douce, R., and Ravanel, S. (1999) *Plant Physiol.* **120,** 1206
8. Eckermann, C., Eichel, J., and Schroder, J. (2000) *Biol. Chem.* **381,** 695–703
9. Zeh, M., Leggewie, G., Hoefgen, R., and Hesse, H. (2002) *Plant Mol. Biol.* **48,** 255–265
10. Isegawa, Y., Watanabe, F., Kitaoka, S., and Nakano, Y. (1994) *Phytochemistry* **35,** 59–61
11. Schröder, G., Eichel, J., Breinig, S., and Schroder, J. (1997) *Plant Mol. Biol.* **33,** 211–222
12. Moffatt, B. A., and Weretilnyk, E. A. (2001) *Physiol. Plant.* **113,** 435–442
13. Horne, D. W., Holloway, R. S., and Wagner, C. (1997) *Arch. Biochem. Biophys.* **343,** 201–206
14. Marobbio, C. M., Agrimi, G., Lasorsa, F. M., and Palmieri, F. (2003) *EMBO J.* **22,** 5975–5982
15. Agrimi, G., Di Noia, M. A., Marobbio, C. M., Fiermonte, G., Lasorsa, F. M., and Palmieri, F. (2004) *Biochem. J.* **379,** 183–190
16. Teyssier, E., Block, M. A., Douce, R., and Joyard, J. (1996) *Plant J.* **10,** 903–912
17. Ravanel, S., Cherest, H., Jabrin, S., Grunwald, D., Surdin-Kerjan, Y., Douce, R., and Rébeillé, F. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 15360–15365
18. Drummond, J. T., Jarrett, J., Gonzalez, J. C., Huang, S., and Matthews, R. G. (1995) *Anal. Biochem.* **228,** 323–329
19. Yeo, E. J., and Wagner, C. (1992) *J. Biol. Chem.* **267,** 24669–24674
20. Awai, K., Marechal, E., Block, M. A., Brun, D., Masuda, T., Shimada, H., Takamiya, K., Ohta, H., and Joyard, J. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 10960–10965
21. Douce, R., Bourguignon, J., Brouquisse, R., and Neuburger, M. (1987) *Methods Enzymol.* **148,** 403–415
22. Douce, R., and Joyard, J. (1982) in *Method in Chloroplast Molecular Biology* (Eldman, M., Hallick, R., and Chua, N. H., eds) pp. 239–256, Elsevier/North-Holland Biomedical Press, Amsterdam
23. Jabrin, S., Ravanel, S., Gambonnet, B., Douce, R., and Rébeillé, F. (2003) *Plant Physiol.* **131,** 1431–1439
24. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
25. Chiu, W., Niwa, Y., Zeng, W., Hirano, T., Kobayashi, H., and Sheen, J. (1996) *Curr. Biol.* **6,** 325–330
26. Abel, S., and Theologis, A. (1994) *Plant J.* **5,** 421–427
27. Heldt, H. W. (1980) *Methods Enzymol.* **69,** 604–612
28. Mourioux, G., and Douce, R. (1981) *Plant Physiol.* **67,** 470–473
29. Bruinsma, J. (1961) *Biochim. Biophys. Acta* **52,** 576–578
30. Corpet, F. (1988) *Nucleic Acids Res.* **16,** 10881–10890
31. Whitfield, C. D., Steers, E. J., Jr., and Weissbach, H. (1970) *J. Biol. Chem.* **245,** 390–401
32. Burton, E., Selhub, J., and Sakami, W. (1969) *Biochem. J.* **111,** 793–795
33. Emanuelsson, O., Nielsen, H., Brunak, S., and von Heijne, G. (2000) *J. Mol. Biol.* **300,** 1005–1016
34. Burton, E. G., and Sakami, W. (1969) *Biochem. Biophys. Res. Commun.* **36,** 228–234
35. Curien, G., Ravanel, S., and Dumas, R. (2003) *Eur. J. Biochem.* **270,** 4615–4627, and references therein
36. Chan, S. Y., and Cossins, E. A. (2003) *Pteridines* **14,** 17–26
37. Wallsgrove, R. M., Lea, P. J., and Miflin, B. J. (1983) *Plant Physiol.* **71,** 780–784
38. Mills, W. R., and Wilson, K. G. (1978) *FEBS Lett.* **92,** 129–132

39. Mills, W. R., Lea, P. J., and Miflin, B. J. (1980) *Plant Physiol.* **65,** 1166–1172
40. Nikiforova, V., Kempa, S., Zeh, M., Maimann, S., Kreft, O., Casazza, A. P., Riedel, K., Tauberger, E., Hoefgen, R., and Hesse, H. (2002) *Amino Acids* (*Vienna*) **22,** 259–278
41. Gallardo, K., Job, C., Groot, S. P. C., Puype, M., Demol, H., Vandekerckhove, J., and Job, D. (2002) *Physiol. Plant.* **116,** 238–247
42. Block, M. A., Tewari, A. K., Albrieux, C., Marechal, E., and Joyard, J. (2002) *Eur. J. Biochem.* **269,** 240–248
43. Cheng, Z., Sattler, S., Maeda, H., Sakuragi, Y., Bryant, D. A., and DellaPenna, D. (2003) *Plant Cell* **15,** 2343–2356
44. Black, M. T., Meyer, D., Widger, W. R., and Cramer, W. A. (1987) *J. Biol. Chem.* **262,** 9803–9807