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Functional Characterization of a Methionine γ-Lyase in Arabidopsis and its Implication in an Alternative to the Reverse Trans-sulfuration Pathway

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Methionine γ-lyase (MGL) catalyzes the degradation of L-methionine to α-ketobutyrate, methanethiol and ammonia. The Arabidopsis (*Arabidopsis thaliana*) genome includes a single gene (At1g64660) encoding a protein (AtMGL) with ~35% identity to bacterial and protozoan MGLs. When overexpressed in *Escherichia coli*, AtMGL allowed growth on L-methionine as sole nitrogen source and conferred a high rate of methanethiol emission. The purified recombinant protein exhibited a spectrum typical of pyridoxal 5′-phosphate enzymes, and had high activity toward L-methionine, L-ethionine, L-homocysteine and seleno-L-methionine, but not L-cysteine. Quantitation of mRNA showed that the AtMGL gene is expressed in aerial organs and roots, and that its expression in leaves was increased 2.5-fold by growth on low sulfate medium. Emission of methanethiol from Arabidopsis plants supplied with 10 mM L-methionine was undetectable (<0.5 nmol $min^{-1} g^{-1}$ FW), suggesting that AtMGL is not an important source of volatile methanethiol. Knocking out the AtMGL gene significantly increased leaf methionine content (9.2-fold) and leaf and root *S*-methylmethionine content (4.7- and 7-fold, respectively) under conditions of sulfate starvation, indicating that AtMGL carries a significant flux in vivo. In Arabidopsis plantlets fed L-[^{35}S]methionine on a low sulfate medium, label was incorporated into protein-bound cysteine as well as methionine, but incorporation into cysteine was significantly (30%) less in the knockout mutant. These data indicate that plants possess an alternative to the reverse trans-sulfuration pathway (methionine→homocysteine→cystathionine→cysteine) in which methanethiol is an intermediate.

Keywords: *Arabidopsis thaliana* — Cysteine — Methionine.

Abbreviations: EST, expressed sequence tag; IPTG, isopropyl-β-D-thiogalactopyranoside; MGL, methionine γ-lyase; PLP, pyridoxal 5′-phosphate; RT–PCR, reverse transcription–PCR; SMM, *S*-methylmethionine.

Introduction

Cysteine is formed from sulfide and *O*-acetylserine via the enzyme *O*-acetylserine(thiol)lyase (Riemenschneider et al. 2005). Cysteine is incorporated into proteins and glutathione, and serves as a sulfur donor for the synthesis of S-containing compounds such as methionine. Like bacteria and fungi, plants have a trans-sulfuration pathway, mediated by cystathionine γ-synthase and cystathionine β-lyase, that converts cysteine to homocysteine via cystathionine, the homocysteine then being used to make methionine (Fig. 1). Methionine is a protein constituent and the precursor of *S*-adenosylmethionine (SAM), the universal methyl donor, and of *S*-methylmethionine (SMM), a major form of sulfur transport in some plants (Bourgis et al. 1999).

Whether—or how—methionine is used for cysteine synthesis in plants has long been controversial. It seems generally to be thought that plants, unlike animals, have little or no capacity to metabolize methionine back to cysteine by a reverse trans-sulfuration pathway due to lack of cystathionine β-synthase and cystathionine γ-lyase, which convert homocysteine to cysteine via cystathionine (Fig. 1) (Giovanelli and Mudd 1971, Datko and Mudd 1984). [The legume *Astragalus pectinatus* may be an exception (Halaseh et al. 1977).] Nutritional experiments with soybean (*Glycine max*) cotyledons and *Catharanthus roseus* cell suspension cultures nevertheless indicate that a major methionine→cysteine route of some kind exists in plants (Schwenn et al. 1983, Holowach et al. 1984). Schwenn et al. (1983) proposed methanethiol as an intermediate in this route in *C. roseus*, but did not demonstrate this definitively.

Methanethiol is emitted by various plants in field environments, e.g. wheat (*Triticum aestivum*), and grassland and saltmarsh species (Rennenberg 1991). Some plants also emit methanethiol in response to exposure to excess sulfur in the form of bisulfide (Saini et al. 1995) or sulfur-containing amino acids. Thus, pumpkin leaves produce methanethiol when treated with L- or D-methionine, or *S*-methyl-L-cysteine (Schmidt et al. 1985), and transgenic tobacco plants that overaccumulate methionine emit methanethiol (Boerjan et al. 1994). However, the enzymatic basis of methanethiol formation from methionine has never been determined and remains a mystery.

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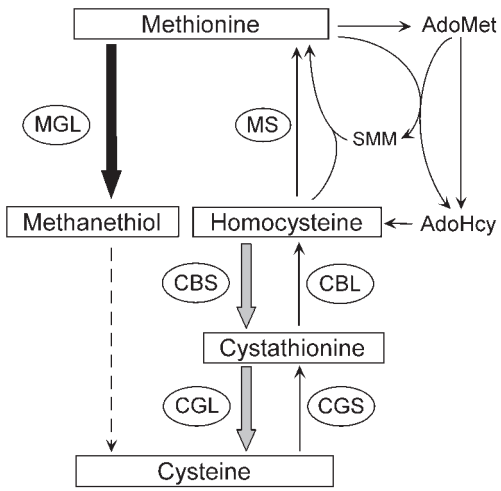

Fig. 1 Scheme showing methionine/cysteine interconversion pathways. Plants, bacteria and fungi have a trans-sulfuration pathway, mediated by cystathionine γ-synthase (CGS) and cystathionine β-lyase (CBL), which converts cysteine to homocysteine via cystathionine. Homocysteine is methylated by methionine synthase (MS) to form methionine. Animals and some microorganisms can metabolize methionine back to cysteine by a reverse trans-sulfuration pathway (broad gray arrows) that involves cystathionine β-synthase (CBS) and cystathionine γ-lyase (CGL). Plants and certain bacteria have been proposed to have an alternative route (or routes) to convert methionine to cysteine, of which the first step is mediated by methionine γ-lyase (MGL, broad black arrow). The subsequent steps in this pathway(s) have not been definitively established (dashed arrows).

Methionine γ-lyase (MGL; EC 4.4.1.11) catalyzes the conversion of L-methionine to α-ketobutyrate, methanethiol and ammonia, and typically attacks other sulfur-containing amino acids such as homocysteine and cysteine. MGL belongs to the γ-subfamily of pyridoxal 5′-phosphate (PLP)-dependent proteins (Christen and Mehta 2001) and has been characterized in bacteria (Kreis and Hession 1973, Ito et al. 1976, Tanaka et al. 1977, Nakayama et al. 1984, Inoue et al. 1995, Faleev et al. 1996, Hori et al. 1996, Dias and Weimer 1998, Manukhov et al. 2005) and protozoans (Lockwood and Coombs 1991, McKie et al. 1998, Tokoro et al. 2003). In the protozoan *Entamoeba histolytica*, which lacks forward and reverse trans-sulfuration pathways, MGL was proposed to be central to sulfur amino acid degradation (Tokoro et al. 2003). It is conceivable that plant MGL could have a similar function, i.e. compensating for the lack of a reverse trans-sulfuration pathway. MGL activity, however, appears never to have been unambiguously demonstrated in plant tissues.

In this work, we identified and characterized an Arabidopsis MGL. We then investigated whether this enzyme is responsible for methanethiol emission from methionine, and whether it participates in a pathway that provides an alternative to reverse trans-sulfuration for converting methionine to cysteine.

Results

Characterization of a cDNA encoding a methionine γ-lyase

Searching the Arabidopsis genome revealed a single gene (At1g64660) encoding an MGL-like protein. The cognate cDNA (obtained from the INRA-Versailles collection) encodes a 441 residue protein (AtMGL) that is 32–37% identical to MGLs from the bacterium *Pseudomonas putida* and the protozoans *Trichomonas vaginalis* and *E. histolytica* (Fig. 2). AtMGL has the Ser-X-X-Lys motif that is highly conserved in MGLs and other members of the γ subfamily of PLP enzymes (Duchange et al. 1983, Belfaiza et al. 1986, Erickson et al. 1990, Lu et al. 1992), the lysine residue being the PLP attachment site (Fearon et al. 1982, Martel et al. 1987, Nakayama et al. 1988). Other residues shown to be important in substrate binding and catalysis in *P. putida* MGL are Tyr114, Asp186 and Arg375 (Inoue et al. 2000, Motoshima et al. 2000), all of which are conserved in AtMGL. Compared with microbial MGLs, AtMGL has an N-terminal extension of ~30 residues that lacks the characteristics of a transit peptide, being hydrophilic and rich in both acidic and basic residues. Searches of expressed sequence tag (EST) databases detected close homologs of the AtMGL cDNA in 12 species of eudicots and monocots, indicating that MGLs are probably ubiquitous among higher plants. Those EST sequences that encoded the N-terminal region of the protein (from eight eudicots and two monocots) all showed hydrophilic, charged extensions like that of AtMGL. Deduced amino acid sequences of AtMGL homologs from two plant species (one Solanaceae and one monocot) are shown in Fig. 2.

Expression of AtMGL enables Escherichia coli to use methionine as sole nitrogen source

Escherichia coli, which does not have an MGL sequence in its genome, grows much faster on methionine as sole nitrogen source when AtMGL is expressed from a plasmid compared with a vector alone control (Fig. 3A) [slow growth of *E. coli* transformed with an empty vector may be due to its ability to use methionine degradation compounds as nitrogen source, as methionine is a photolabile compound (Cohen and Ojanpera 1975, Nakamura et al. 1981)]. This finding shows that AtMGL can make available the amino group of methionine, presumably via its release as ammonia in the MGL reaction. Consistent with this explanation, the culture plates had the characteristic odor of methanethiol. To confirm the latter observation, we measured methanethiol emission rates of *E. coli* cells expressing the AtMGL cDNA, or harboring the vector

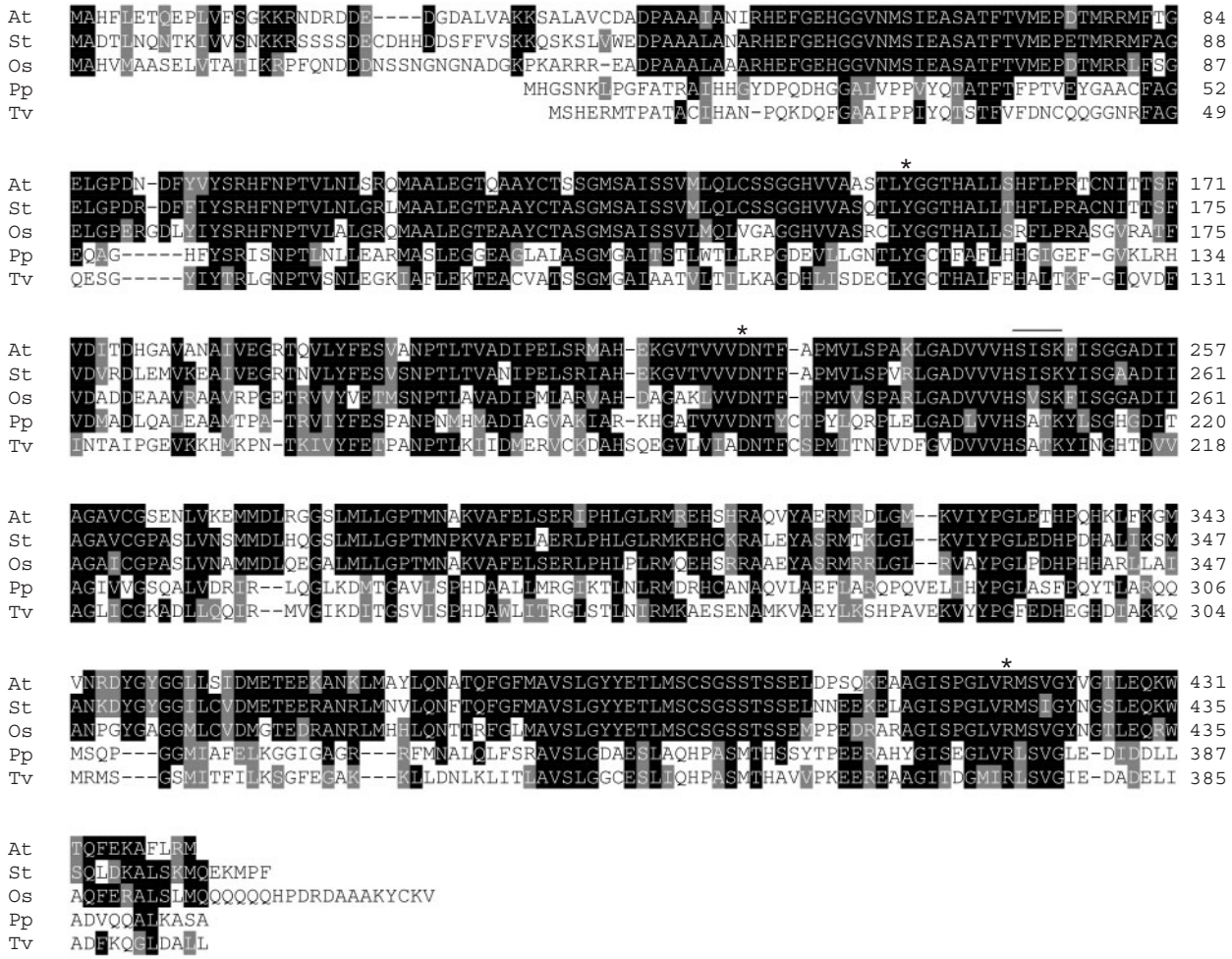

Fig. 2 Amino acid sequence alignment of MGLs from Arabidopsis, *Solanum tuberosum* (GenBank accession Nos. CK255447 and CK255448), *Oryza sativa* (GenBank accession No. AK100465), *Trichomonas vaginalis* (GenBank accession No. CAA04124) and *Pseudomonas putida* (GenBank accession No. P13254). The overline marks the S-X-X-K motif; asterisks indicate other conserved residues shown to be important in substrate binding and catalysis in the γ subfamily of PLP-dependent enzymes.

alone, when grown in liquid minimal medium containing methionine or ammonium as the only nitrogen source. *Escherichia coli* transformants harboring the vector alone could grow on medium containing methionine as the only nitrogen source (as explained above on solid medium), but at a rate ~3 times lower than *E. coli* cells expressing the AtMGL cDNA (data not shown). Methanethiol emission was detected only from cells expressing the AtMGL cDNA and grown on methionine (Fig. 3B). Dimethyldisulfide, an oxidation product of methanethiol, was not detected (<0.01 nmol h^{-1} per 10^{10} cells) from any of the treatments (data not shown). These results show that AtMGL catalyzes the conversion of methionine to methanethiol and ammonia in vivo.

Biochemical characterization of recombinant AtMGL

The AtMGL coding sequence was cloned into the pET-43.1a expression vector with or without a 3′-terminal hexahistidine tag, and expressed in *E. coli*. Measurements of MGL activity in desalted total protein extracts showed that native and tagged AtMGL gave the same MGL activity (56 nmol min^{-1} mg^{-1} protein). Further analyses were therefore done with the histidine-tagged enzyme after purification on an Ni^{2+} resin column. The preparation was ≥95% homogeneous, as judged from Coomassie-stained SDS–polyacrylamide gels (Fig. 4A). The apparent molecular mass of the histidine-tagged AtMGL (48 kDa) agreed well with the predicted value (48.8 kDa). The purified protein had a specific activity of 0.4 μmol min^{-1} mg^{-1} with L-methionine as substrate, which is at the low end of the range (0.26–45 μmol min^{-1} mg^{-1}) reported for microbial MGLs (Ito et al. 1976, Nakayama et al. 1984, Lockwood and Coombs 1991, Faleev et al. 1996, Hori et al. 1996, Tan et al. 1997, Dias and Weimer 1998, McKie et al. 1998, Tokoro et al. 2003, Manukhov et al. 2005). The purified protein was quite unstable, losing >80% of its activity after

1 month at −80°C in 10% glycerol or at 4°C in 40% glycerol.

Size exclusion chromatography of the purified AtMGL gave a molecular mass of 212 kDa, indicating that it exists as a homotetramer, as do most other MGLs (Ito et al. 1976, McKie et al. 1998, Tokoro et al. 2003). The protein exhibited an absorption maximum at 422 nm (Fig. 4B), which is typical of PLP-dependent proteins. After alkalization with NaOH, the peak at 422 nm shifted to 388 nm, indicating that all the PLP was released. Spectrophotometric quantification of the PLP (Peterson and Sober 1954) gave a value of 0.88 mol PLP mol^{-1} of subunit.

Tests of compounds that are substrates for other MGLs showed that AtMGL catalyzed α,γ-elimination reactions on various methionine derivatives, and—less efficiently—α,β-eliminations on cystine and *O*-acetylserine. Cysteine was not attacked, however (Table 1). Comparison of the biochemical characteristics of four good substrates (L-methionine, L-ethionine, L-homocysteine and seleno-L-methionine) (Table 2) showed that AtMGL had the best catalytic efficiency (K_{Cat}/K_m) towards L-ethionine, but differences between substrates were fairly modest.

Expression of AtMGL mRNA

Real-time quantitative reverse transcription–PCR (RT–PCR) showed that AtMGL mRNA was expressed in all organs tested, 5–10 times more strongly in roots, stems and siliques than in leaves (Fig. 5A). These results should, however, be treated with caution because roots were grown hydroponically while other organs were harvested from plants grown in potting soil. Levels ranged from 0.00007 to 0.0008% of total RNA, which corresponds to an mRNA frequency from 1 in ~15,000 to 1 in ~1,500, assuming that mRNAs constitute 1% of total RNA. A similar pattern of ubiquitous, low to moderate expression is also reported for the AtMGL gene in the GENEVESTIGATOR Arabidopsis microarray database (Zimmermann et al. 2004).

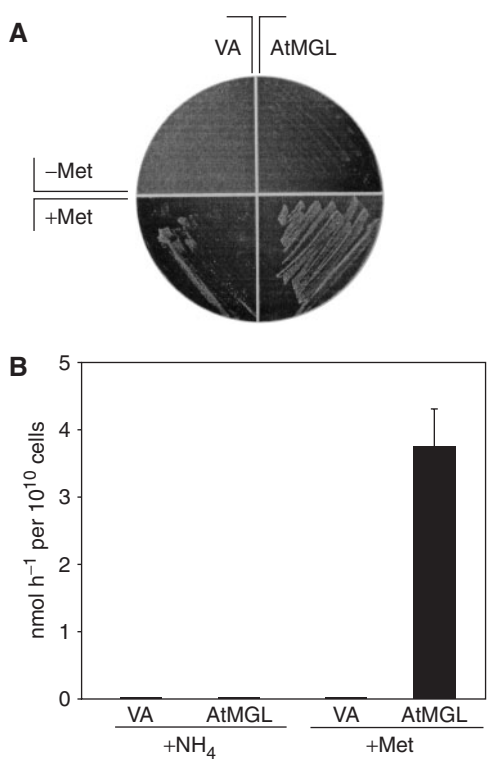

Fig. 3 Functional characterization of AtMGL in vivo. (A) Functional expression of AtMGL in *E. coli* Rosetta™ (DE3) cells harboring pET-43.1a alone (VA) or containing the AtMGL cDNA (AtMGL). Cells were grown at 25°C for 7 d on minimal medium plates without any nitrogen source (–Met), or with methionine (+Met) as nitrogen source. (B) Emission of methanethiol from *E. coli* Rosetta™ (DE3) cells harboring pET-43.1a alone (VA) or containing the AtMGL cDNA (AtMGL). Cells were grown in liquid minimal medium with ammonium ($+NH_4$) or methionine (+Met) as sole nitrogen source.

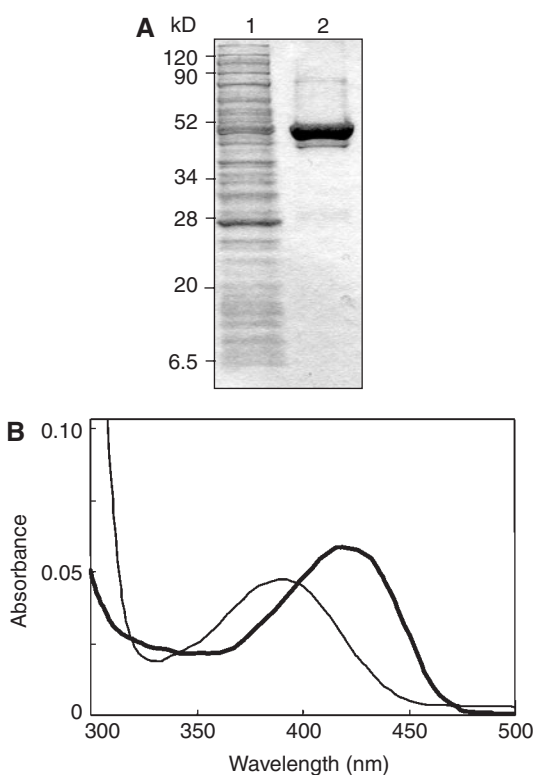

Fig. 4 Purification of recombinant AtMGL and evidence for a PLP cofactor. (A) SDS–PAGE of histidine-tagged AtMGL isolated by Ni^{2+} chelate affinity chromatography. The gel was stained with Coomassie blue. Lane 1 was loaded with 5 µg of protein from the fraction not bound by the resin, and lane 2 was loaded with 6 µg of purified protein. The positions of molecular mass markers are indicated. (B) The absorption spectrum of purified AtMGL (0.39 mg ml^{-1}) in 50 mM Na phosphate, pH 8.0 (broad line) and after addition of NaOH to a final concentration of 0.1 M (narrow line).

AtMGL gene expression was 2.5-fold higher in leaves from plantlets grown on low sulfate medium compared with normal sulfate medium (significant at $P<0.01$) (Fig. 5B). Spraying leaves or treating roots with methionine or homocysteine did not affect AtMGL expression (data not shown).

Isolation and characterization of an AtMGL mutant

To assess the physiological significance of AtMGL, we identified a T-DNA insertion mutant of Arabidopsis (SALK_040380) in the Salk Institute collection via the sequence flanking the insert. Resequencing of this region confirmed the presence of an insert near the 5′ end of the second exon (Fig. 6A). This insertion created at least two in-frame stop codons and so is predicted to result in a complete knockout. Plants homozygous for the mutation and their wild-type siblings were identified by PCR and subjected to Southern analysis using a T-DNA sequence as probe (Fig. 6B). Only the mutant plants gave hybridizing bands, establishing that the T-DNA is inserted only at the AtMGL locus. The multiple banding pattern indicates that several concatenated T-DNA copies are present at this locus. Real-time quantitative RT–PCR analysis showed that AtMGL mRNA levels in the mutant were <4% of those in the wild type (data not shown). There were no discernible differences in growth, morphology, leaf color or fertility between mutant and wild-type plants.

Table 1 Relative activity of AtMGL toward various substrates

Substrate	Relative activity (%)
L-Methionine	100
L-Ethionine	313
L-Homocysteine	209
Seleno-L-methionine	107
L-Cystine	33
S-Adenosyl-L-methionine	30
L-Methionine sulfone	19
L-Methionine sulfoxide	14
L-Cystathionine	11
O-Acetyl-L-serine	9
L-Methionine-DL-sulfoximine	<3
D-Methionine	<3
S-Methyl-L-methionine	<3
L-Cysteine	<3
S-Methyl-L-cysteine	<3
S-Adenosyl-L-homocysteine	<3
L-Homoserine	<3

Assays (50 µl) contained 8.8 µg of purified AtMGL protein and 10 mM amino acid. Incubation time was 20 min. α-Ketoacids were quantified colorimetrically as 3-methyl-2-benzothiazolone hydrazone derivatives. Data are expressed relative to the activity with L-methionine as substrate (0.42 nmol min^{-1} mg^{-1} protein), and are based on three replicates.

Effects of the mutation on methanethiol emission and amino acid levels

Wild-type and mutant plants grown in soil, and leaf disks floated for up to 48 h on 10 mM L-methionine solution, did not emit detectable methanethiol or dimethyldisulfide (<0.5 nmol min^{-1} g^{-1} FW; data not shown). Such emissions were also not detected from roots grown axenically in liquid medium containing 10 mM L-methionine. These results imply either that AtMGL is inactive in vivo, or that it is active, with the methanethiol it produces being further metabolized rather than emitted. Analysis of free amino acids favored the latter alternative because mutant plantlets grown on low sulfate medium showed a 9.2-fold increase in methionine level in leaves, and 4.7- and 7-fold increases in SMM level in leaves and roots, respectively (significant at $P<0.05$) (Table 3). There were differences in the levels of other amino acids (Table 3), but the fold increases in methionine and SMM levels were significantly higher. There was no significant difference between the wild type and the mutant grown on normal sulfate medium (data not shown). As SMM is freely interconvertible with methionine (Mudd and Datko 1990, James et al. 1995, Bourgis et al. 1999), its accumulation in the mutant, in addition to methionine accumulation itself, signals a build up of methionine moieties. This build up suggests that AtMGL normally mediates a significant flux in vivo.

Table 2 Kinetic constants of AtMGL

Substrate	K_m(mM)	V_{max}(nmol min^{-1} mg^{-1})	K_{Cat}(min^{-1})	K_{Cat}/K_m(min^{-1} mM^{-1})
L-Methionine	72	194	9.5	0.13
L-Ethionine	14	182	8.9	0.63
L-Homocysteine	92	743	36.2	0.39
Seleno-L-methionine	40	238	11.6	0.29

Assays (50 µl) contained 8.8 µg of purified recombinant AtMGL protein. Incubation time was 20 min at 30°C. α-Ketoacids were quantified colorimetrically as 3-methyl-2-benzothiazolone hydrazone derivatives. K_m and V_{max} values were estimated by fitting substrate concentration and velocity values to the Michaelis–Menten equation using non-linear regression. Data are means of three independent determinations.

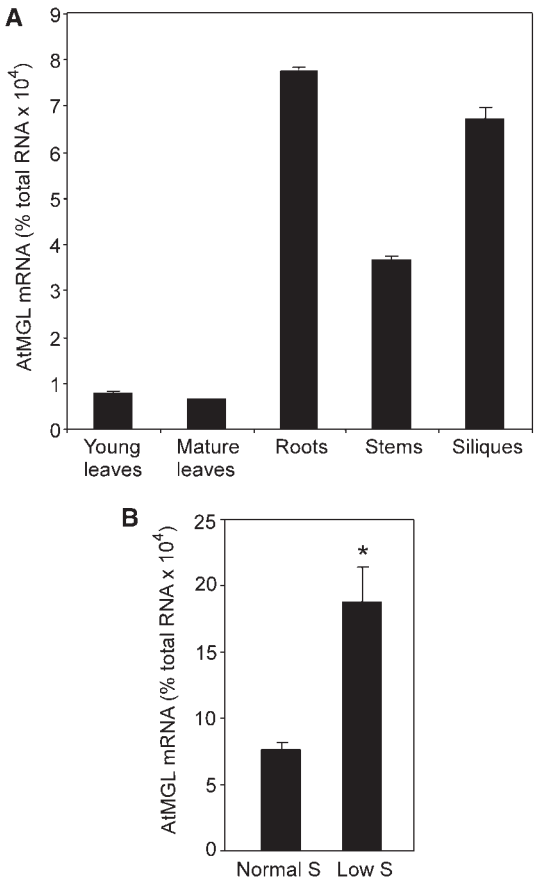

Fig. 5 Quantification of AtMGL mRNA in Arabidopsis organs. Levels of mRNA were determined by real-time quantitative RT–PCR, using amplicons spanning two exons of the gene. (A) Young leaves were harvested at the 4 to 6 leaf stage, mature leaves at the start of bolting, and stems and developing siliques before flowering had ceased. Roots were from hydroponically grown plants, harvested at 5 weeks before flowering had ceased. Three independent RNA extracts were made of each organ, and triplicate mRNA determinations were made on each extract. Data are means and SE of all nine determinations. The mRNA level in each sample was expressed as a percentage of the weight of total RNA. Internal RNA standards were used to estimate recovery from RT–PCR of each sample; the data shown are corrected for recovery. (B) Leaves from Arabidopsis plantlets grown on normal or low sulfate solidified medium were harvested at the rosette stage. Four to six independent RNA extracts were made for each condition, and triplicate mRNA determinations were made on each extract. Data are means and SE of all 12 or 18 determinations. The mRNA level in each sample was expressed as a percentage of the weight of total RNA. The asterisk indicates a significant ($P<0.01$) difference between normal and low sulfate medium.

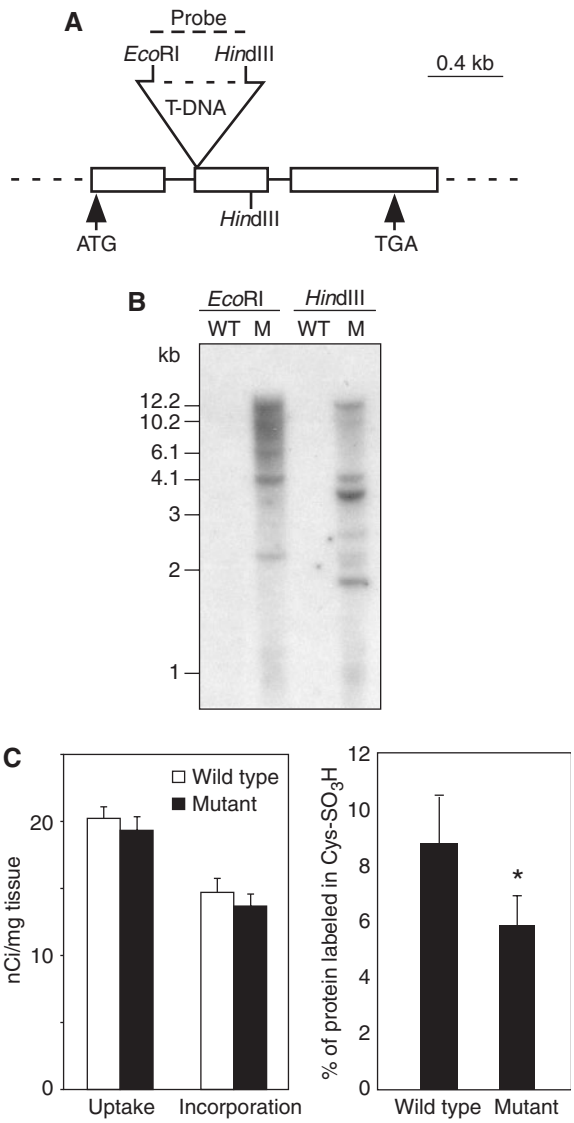

Fig. 6 Characterization of a T-DNA mutant of AtMGL. (A) Scheme of the structure of the MGL gene, with introns as solid lines and exons as boxes. The positions of the T-DNA insertion, of the start and stop codons, and of *Eco*RI and *Hin*dIII restriction sites are indicated. (B) Southern blot analysis of mutant homozygotes (M) and their wild-type siblings (WT). The ^{32}P-labeled probe was specific for the T-DNA sequence. Genomic DNA (5 µg lane^{-1}) was digested with *Eco*RI or *Hin*dIII. The positions of size markers are shown on the left. (C) Methionine to cysteine metabolism in Arabidopsis wild-type and mutant plants. Plants were grown for 26 d on a low sulfate medium containing a trace amount of L-[^{35}S]methionine. Proteins were extracted, oxidized by performic acid, and hydrolyzed. Labeled cysteic acid (Cys-SO_3H) was isolated by ion exchange chromatography and thin-layer electrophoresis, and quantified by scintillation counting. Three independent experiments were carried out and duplicate determinations were made for each. Data are expressed as the percentage of total protein label recovered as cysteic acid, and are means and SE of all six determinations. The asterisk indicates a significant ($P<0.05$) difference between wild type and mutant.

Table 3 Free amino acid pool sizes (nmol g^{-1} FW) in leaves and roots of wild-type and AtMGL mutant plants grown on low sulfate medium

Compound	Leaves		Roots	
	Wild type	Mutant	Wild type	Mutant
Methionine	0.3 ± 0.0	3.2 ± 0.2*	ND	ND
SMM	1.0 ± 0.2	4.5 ± 0.5*	2.9 ± 0.6	20.4 ± 4.0*
Aspartate	23.8 ± 2.5	27.5 ± 6.0	140.7 ± 10.3	134.5 ± 27.6
Glutamate	30.5 ± 3.1	42.5 ± 7.8	268.4 ± 14.3	282.4 ± 54.5
Serine	59.2 ± 4.3	117.3 ± 11.0*	1156.4 ± 310.9	1296.9 ± 231.0
Asparagine	39.8 ± 2.4	81.1 ± 7.4*	ND	ND
Glycine	47.0 ± 3.9	77.6 ± 4.8*	317.8 ± 69.1	294.3 ± 63.2
Threonine	14.6 ± 1.1	31.2 ± 2.1*	501.7 ± 109.1	522.6 ± 71.1
Arginine	66.3 ± 13.1	78.6 ± 11.6	101.7 ± 12.0	127.0 ± 21.4
Alanine	16.5 ± 1.7	31.0 ± 4.8*	278.2 ± 46.0	274.5 ± 32.5
Proline	62.6 ± 6.4	153.8 ± 21.3*	389.7 ± 170.9	480.8 ± 231.1
Tyrosine	2.0 ± 0.1	5.7 ± 0.4*	53.3 ± 6.0	46.2 ± 2.7
Valine	5.5 ± 0.5	15.2 ± 1.4*	167.2 ± 32.4	233.7 ± 32.8
Isoleucine	2.7 ± 0.2	7.6 ± 0.8*	64.4 ± 14.7	80.3 ± 11.8
Leucine	1.6 ± 0.1	6.0 ± 0.5*	54.0 ± 13.2	77.4 ± 16.6
Lysine	2.3 ± 0.4	3.9 ± 1.2	34.9 ± 23.4	41.9 ± 5.7
Phenylalanine	6.2 ± 0.4	12.7 ± 1.3*	118.4 ± 7.8	119.7 ± 14.6

Data are means ± SE for 4–6 independent samples. Asterisks indicate significant ($P < 0.05$) differences between the wild type and mutant. ND, not determined.

Methionine to cysteine conversion in wild-type and mutant plants

If Arabidopsis plants metabolize methanethiol as the above results imply, a prime candidate for its fate is cysteine because there is persuasive nutritional evidence that plants can convert methionine to cysteine (Schwenn et al. 1983, Holowach et al. 1984), and methanethiol has been proposed as an intermediate (Schwenn et al. 1983). Therefore, we measured the capacity of Arabidopsis to metabolize methionine to cysteine and checked whether this process was impaired in the AtMGL mutant. Plantlets were grown for 26 d on a low sulfate medium containing L-[^{35}S]methionine. Soluble proteins were extracted, performic acid-oxidized, hydrolyzed, and methionine sulfone and cysteic acid, the oxidation products of methionine and cysteine, respectively, were analyzed by thin-layer electrophoresis. As shown in Fig. 6C, ^{35}S uptake and incorporation into protein were similar in wild-type and mutant plants. Consistent with the operation of a methionine→cysteine route, 8.8% of total protein label was recovered as cysteic acid in the wild type. The corresponding value for the mutant (5.8%) was significantly ($P < 0.05$) lower. This result implicates AtMGL in the methionine to cysteine conversion process.

Discussion

Our results establish that Arabidopsis has an active MGL, and EST data imply that this enzyme is widespread in plants. Like its bacterial and protozoan homologs, plant MGL is a PLP-dependent enzyme comprised of four identical subunits that catalyzes the degradation of L-methionine and related compounds. That the Arabidopsis gene is expressed constitutively in aerial organs and roots indicates that AtMGL has a housekeeping function in plants.

Our data further suggest what this housekeeping function is, namely to mediate the first step in methionine to cysteine conversion via methanethiol, as hypothesized by Schwenn et al. (1983). Such a methanethiol pathway, which provides an alternative to the reverse transsulfuration pathway, operates in certain bacteria (Vermeij and Kertesz 1999, Seiflein and Lawrence 2001). The elevated K_m of AtMGL for methionine might seem to argue against such a function. However, while this paper was under review, a study on MGL in Arabidopsis cells also reported a high K_m for this enzyme (≈10 mM) (Rébeillé et al. 2006). The authors observed that MGL expression was induced in response to high methionine levels in the medium and proposed that this induction could compensate for the high K_m of the enzyme. The induction of AtMGL in plants grown under low S conditions could likewise compensate for the high K_m. It should also be noted that our kinetics measurements were made with a very unstable enzyme that had lost >80% of its original activity, so that partial denaturation could have raised the K_m.

At first sight, it is surprising that so much (8.8%) of the protein-bound label in [^{35}S]methionine-fed wild-type plants was present as cysteine, because this implies that the methionine→cysteine flux is a major one. However, a major flux is entirely consistent with classical nutritional experiments, which demonstrated that plant cells or tissues can use methionine as sole sulfur source (Schwenn et al. 1983, Holowach et al. 1984). Moreover, our experiments were done in a low sulfate medium, which may have promoted the salvage of methionine for re-use in cysteine synthesis as sulfate became depleted.

It was also at first surprising that we did not detect methanethiol emission ($<0.5\,nmol\,min^{-1}\,g^{-1}$ FW) from methionine-fed Arabidopsis tissues, given the characteristic odor of methanethiol emitted from methionine-fed Arabidopsis cells (Rébeillé et al. 2006) and the high emission rates reported for methionine-treated pumpkin leaves ($\sim 10\,nmol\,min^{-1}\,g^{-1}$ FW; Schmidt et al. 1985). However, it is noteworthy that whereas methionine-overaccumulating tobacco plants emit copious methanethiol and have a characteristic odor (Boerjan et al. 1994), no such odor has been reported for various methionine-overproducing (*mto*) Arabidopsis mutants (Inaba et al. 1994, Bartlem et al. 2000, Shen et al. 2002). Perhaps Arabidopsis tissues (as opposed to cultured cells) are efficient at metabolizing methanethiol, so that little of it is lost through volatilization.

Knocking out the AtMGL gene significantly reduced methionine to cysteine conversion, but by no means abolished it. Several possibilities could account for the large residual methionine→cysteine flux (besides the remote one that Arabidopsis has another, still unrecognized MGL gene). First, notwithstanding the prevailing view that plants lack reverse trans-sulfuration, Arabidopsis may have proteins with the required activities (cystathionine β-synthase and cystathionine γ-lyase), as reported for the legume *Astragalus pectinatus* (Halaseh et al. 1977). Secondly, amino acid oxidase or transaminase could have converted methionine to α-ketomethiolbutyrate, which could have given rise to methanethiol as occurs in *Lactococcus lactis* (Bonnarme et al. 2004). Thirdly, while we can be sure that [^{35}S]cyst(e)ine was not a major contaminant of the [^{35}S]methionine supplied (analysis confirmed that [^{35}S]cysteine contamination was <0.5%), we cannot exclude the possibility that the [^{35}S]methionine in the medium underwent chemical or photochemical oxidation during the experiment, giving rise to products that were assimilated into cysteine. Methionine is well known to be photolabile (Cohen and Ojanpera 1975, Nakamura et al. 1981).

Finally, how methanethiol is used for cysteine synthesis remains unclear. Rébeillé et al. (2006) showed that methanethiol produced by the MGL-catalyzed reaction in Arabidopsis cells could react with an activated form of serine to produce *S*-methylcysteine. However, they could not identify metabolites of *S*-methylcysteine and showed that *S*-methylcysteine produced in the cytoplasm was rapidly transferred to the vacuole and could play a storage role. Therefore, such a pathway seems unlikely to lead to cysteine formation. For cells of the plant *C. roseus*, Schwenn et al. (1983) proposed an *S*-methyl exchange between methanethiol and homocysteine, releasing H_2S for assimilation into cysteine. For the bacterium *P. putida*, Vermeij and Kertesz (1999) proposed that methanethiol is metabolized via methanesulfonate, sulfonate and sulfide. As the evidence for both these pathways is indirect, they remain to be definitively established. More generally, by corroborating the intriguing results of half-forgotten nutritional experiments, our data underscore that much remains to be learned about plant methionine metabolism.

Materials and Methods

Chemicals and reagents

L-[^{35}S]Methionine ($1{,}000\,Ci\,mmol^{-1}$) and [α-^{32}P]dCTP ($3{,}000\,Ci\,mmol^{-1}$) were from PerkinElmer Life Sciences. Amino acids and other chemicals were from Sigma or Fisher. AG-50 (H^+) ion exchange resin was from Bio-Rad. Cellulose (0.1 mm) plates were from Merck.

Plants and growing conditions

Arabidopsis thaliana plants (ecotype Columbia) were grown at 23–28°C in 12 h days (photosynthetic photon flux density $80\,\mu E\,m^{-2}\,s^{-1}$) in potting soil irrigated with water; when roots were required, plants were grown in hydroponic culture as described by Gibeaut et al. (1997).

cDNA isolation and expression in Escherichia coli

EST GenBank™ accession number Z34674 encoding AtMGL was obtained from INRA (Versailles, France), sequenced, and cloned into pET-43.1a (Novagen, Madison, WI, USA) as follows. Expand high fidelity *Taq* DNA polymerase (Roche, Indianapolis, IN, USA) was used to amplify the cDNA using the primers 5′-AAAACATATGGCTCATTTCCT-3′ (forward) and 5′-AAAAGTCGACTTACATTCTGAGGAA-3′ (reverse) or 5′-AAAAGTCGACCATTCTGAGGAATGCT-3′ (reverse for addition of the hexahistidine tag). The resulting amplicon was digested with *Nde*I and *Sal*I and cloned into pET-43.1a digested with *Nde*I and *Xho*I. These constructs were electroporated into *E. coli* DH10B cells, verified by sequencing and electroporated into *E. coli* Rosetta™ (DE3) cells (Novagen). For enzyme production, cells were grown at 37°C in LB medium containing $100\,\mu g\,ml^{-1}$ ampicillin and $34\,\mu g\,ml^{-1}$ chloramphenicol until A_{600} reached 0.6. Isopropyl-D-thiogalactopyranoside (IPTG) was then added (final concentration 100 μM) and incubation continued for 18 h at 25°C.

Utilization of methionine as nitrogen source by E. coli overexpressing AtMGL

Transformed *E. coli* Rosetta™ cells were plated on M9 minimal medium (Sambrook et al. 1989) minus NH_4Cl, containing trace elements (Neidhardt et al. 1974), 0.4% glucose, 1.5% agar, $100\,\mu g\,ml^{-1}$ ampicillin, $34\,\mu g\,ml^{-1}$ chloramphenicol, 100 μM IPTG,

with or without 20 mM L-methionine as nitrogen source (filter sterilized, added after autoclaving). Growth was at 25°C. For methanethiol emission measurement experiments, *E. coli* cells overexpressing AtMGL were grown overnight in 50 ml of LB medium containing 100 μg ml^{-1} ampicillin and 34 μg ml^{-1} chloramphenicol. After harvesting by centrifugation, cells were washed in 25 ml of 1 mM $MgSO_4$ and 0.1 mM $CaCl_2$. This step was repeated twice. The final pellet was resuspended in 5 ml of 1 mM $MgSO_4$ and 0.1 mM $CaCl_2$. An aliquot was used to inoculate 100 ml (in 250 ml flasks) of either M9 minimal medium or M9 minimal medium minus NH_4Cl supplemented with 20 mM L-methionine, containing trace elements, 0.4% glucose, 100 μg ml^{-1} ampicillin, 34 μg ml^{-1} chloramphenicol and 100 μM IPTG. Cells were grown for up to 168 h at 25°C, and headspace gases were trapped by closing the flasks with a serum cap for periods up to 48 h. Headspace samples of 1 ml were injected onto a 210 × 0.3 cm stainless steel column packed with 80/100-mesh Porapak Q (Supelco) in a Hewlett-Packard 5890 series II gas chromatograph equipped with a flame ionization detector. Column temperature was set at 145°C for 6 min, then raised to 230°C with a ramp of 8.5°C min^{-1}, and held for 2 min. Products were quantified by peak area and identified by comparison of their retention times with those of authentic methanethiol and dimethyldisulfide.

Enzyme assays

The activity of AtMGL toward various substrates was measured by monitoring α-ketoacid production as described by Soda (1968). Assays (final volume 50 μl) were carried out at 30°C in 50 mM Na-phosphate, pH 8.0, containing 0.1 mM PLP. Substrates were used at a concentration of 10 mM. The reaction was stopped by adding 25 μl of 4.5% trichloroacetic acid. A 25 μl aliquot was transferred to a new tube containing 25 μl of 0.05% 3-methyl-2-benzothiazolone hydrazone, and the mixture was incubated for 30 min at 50°C. The absorbance at 335 nm was measured, and α-ketoacid content was estimated from a calibration curve made with α-ketobutyrate.

Protein purification and molecular mass determination

Operations were at 0–4°C. AtMGL-expressing Rosetta™ cells from a 1 liter culture were harvested by centrifugation, resuspended in 20 ml of 50 mM Na-phosphate, pH 8.0, 10 mM imidazole, 1.2 mM β-mercaptoethanol, 0.5 M NaCl, 20 μM PLP, and broken in a Mini-BeadBeater (Biospec Products, Bartlesville, OK, USA) using 0.1 mm zirconia/silica beads. Protein purification by Ni^{2+}-affinity chromatography under native conditions followed the manufacturer's protocol (Qiagen). Native molecular mass was estimated using a Superdex 200 HR 10/30 column (Amersham Biosciences) equilibrated in 50 mM Na-phosphate, pH 8.0, 150 mM NaCl; reference proteins were thyroglobulin, apoferritin, β-amylase, bovine serum albumin and carbonic anhydrase. Denaturing electrophoresis was on 12.5% polyacrylamide gels. Protein was estimated by the method of Bradford (1976) using bovine serum albumin as standard.

Arabidopsis mutant

A line (SALK_040380) containing a T-DNA insertion in the AtMGL gene was identified in the Salk Institute collection (ecotype Columbia). Segregants wild type or homozygous for the mutation were identified by PCR using gene-specific primers located 5′ or 3′ of the T-DNA insertion (5′-ATAGCGAATATCC GACATGAGT-3′ and 5′-CACCACATCTG-CTCCAAGCT-3′, respectively) and the T-DNA-specific primer 5′-GCGTGGACC GCTTGCTGCAACT-3′. DNA was extracted by the 'Shorty' protocol available on the University of Wisconsin Biotechnology Center website. The insertion site was confirmed by sequencing.

Gel blot analyses

Genomic DNA was isolated as described (Lassner et al. 1989) from 2 g of leaves pooled from 20 plants, digested, separated by 0.8% agarose gel electrophoresis (5 μg lane^{-1}), and blotted to a Protran® membrane. Blots were hybridized as above and washed in 0.1× SSC, 0.5% SDS at 37°C. The probe was a 1,077 bp fragment of the pROK2 vector (Baulcombe et al. 1986) digested with *Eco*RI and *Hin*dIII. The probe was labeled with [α-^{32}P]dCTP by the random primer method. Hybridization was detected by autoradiography.

Real-time quantitative RT–PCR

Total RNA was extracted from at least three samples of each tissue using RNeasy kits (Qiagen, Valencia, CA, USA) or Absolutely RNA® purification kits (Stratagene, La Jolla, CA, USA) and treated with DNase (DNA-*free*™ kit, Ambion, Austin, TX, USA). Real-time quantitative RT–PCR was performed on 250 ng of RNA in 25 μl reactions using either TaqMan® One-Step RT–PCR Master Mix Reagents (Applied Biosystems, Foster City, CA, USA) and an Applied Biosystems GeneAmp 5700 sequence-detection system, or Brilliant® SYBR® Green QRT-PCR Reagents (Stratagene, La Jolla, CA, USA) and a Stratagene Mx3005P™ QPCR system. The primers and probe (designed with Applied Biosystems Primer Express software) were as follows: forward primer 5′-CAACCTCAGCCGCCAGAT-3′; reverse primer 5′-TCGCCGACATACCGCTAGA-3′; probe 5′-CTCGAAGGC ACCCAAGCTGCCTACT-3′ with the fluorescent reporter dye 6-carboxyfluorescein and the quencher dye 6-carboxytetramethylrhodamine bonded to the 5′ and 3′ end, respectively. The amplicon was 75 bp long. RT–PCR conditions were as follows: 48°C for 30 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The standard was sense-strand RNA, prepared as described (Rontein et al. 2003). The template for in vitro transcription was the pHD-1 vector containing the Z34674 EST, linearized with *Nde*I. Samples and standards were run in duplicate. A C_T threshold value was determined from amplification curves by selecting an optimal ΔRn (emission of the reporter dye over starting background fluorescence) in the exponential part of the plots.

Methionine metabolism by Arabidopsis

Plants were grown vertically for 26 d on 40 ml of low sulfate solidified medium (in 9 × 2 cm plastic plates) containing 0.5% (w/v) Phytagel (Sigma), 1% (w/v) sucrose, Gamborg B5 medium vitamins (Gamborg et al. 1968), MS medium salts modified by replacing sulfate salts with the corresponding chloride salts except for $MgSO_4$ (30 μM), and 20 μCi of L-[^{35}S]methionine. Seven plants were grown per plate. Plants were ground in liquid N_2 and extracted in 0.2–0.4 ml of 30 mM NH_4HCO_3, pH 8.0, containing 2.5 mg ml^{-1} bovine serum albumin, for 20 min at 0°C with periodic gentle agitation. After centrifuging to clear, the extract was mixed with 5 vols. of ice-cold acetone, held on ice for 1 h, and recentrifuged. The pellet was washed with 80% acetone, dried, redissolved in 2 ml of a performic acid solution (Moore 1963), and incubated for 5–8 h at 0°C. The oxidation was stopped by adding 0.3 ml of 48% HBr. The sample was dried in vacuo at 35°C, redissolved in 3 ml of 6 M HCl, and heated at 110°C for 18 h. After lyophilizing, the hydrolysate was dissolved in 0.4 ml of water and applied to a 1 ml AG 50 (H^+) resin. The column was washed with 5 ml of water to recover cysteic acid, and eluted with 5 ml of

3 M NH_4OH to recover methionine sulfone. To quantify the ^{35}S content of cysteic acid and methionine sulfone, aliquots of the wash and eluate were subjected to thin-layer electrophoresis on cellulose plates for 10 min at 1.8 kV in pyridine : acetic acid : water (1 : 1 : 38, v/v/v) at 4°C. Cysteic acid and methionine sulfone zones were located autoradiographically and by reference to standards, scraped from the plates, and quantified by scintillation counting. Cysteine contamination of the L-[^{35}S]methionine supplied was determined by this method to be <0.5%.

Amino acid and SMM analyses

Arabidopsis plants were grown on either normal or low sulfate solidified medium for 7 weeks as described above. Leaf and root tissues (~160 mg) were frozen in liquid N_2, lyophilized, weighed and pulverized. The resulting powder was extracted by shaking with 0.05–0.07 ml of 10 mM HCl and 0.5 ml of $CHCl_3$. γ-Aminobutyric acid was added as internal standard. For HPLC, 10 µl of the aqueous phase was derivatized with AccQ-Fluor™ reagent (6-aminoquinolyl-*N*-hydroxysuccinimidylcarbamate; Waters, Milford, MA, USA) in a final volume of 100 µl, and a 15 µl aliquot was analyzed by HPLC-fluorescence according to Waters' recommendations. Amino acid and SMM HPLC analyses were performed as described (Kim et al. 2002) with an improved separation program (Dr. T. Leustek personal communication). Three buffers were used: A, sodium acetate and triethylamine (Waters; pH adjusted to 5.5 with NaOH); B, acetonitrile : water (30 : 70); and C, acetonitrile : water (60 : 40) by volume. The improved elution program was: 0–0.5 min 100% A; 0.5–1.5 min linear gradient to 6.2% B; 1.5–32 min linear gradient to 7.3% B; 32–51 min linear gradient to 28% B; 51–66 min linear gradient to 37% B; 66–84 min linear gradient to 65% B; 84–97 min linear gradient to 100% B; 97–101 min linear gradient to 50% C; and finally 101–104 min linear gradient to 100% C.

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