Plant Biotechnology 22, 379–388 (2005)

Review

Dynamics of methionine biosynthesis

Derek B. Goto[1], Hitoshi Onouchi[2], Satoshi Naito[2]*

[1] Research Institute for Electronic Science, Hokkaido University, Sapporo 060-0812, Japan; [2] Graduate School of Agriculture, Hokkaido University, Sapporo, 060-8589, Japan
*E-mail: natio@abs.agr.hokudai.ac.jp Tel: +81-11-706-2800 Fax: +81-11-706-4932

Received August 31, 2005; accepted September 12, 2005 (Edited by K. Yazaki)

Abstract Methionine biosynthesis in plants provides the primary source of this essential amino acid. Early pioneering works characterised many of the steps involved in the methionine biosynthetic pathway and properties of the enzymes involved. They also showed that methionine biosynthesis was strictly controlled in higher plants and part of a larger, complex pathway that involves the biosynthesis of lysine, threonine and isoleucine. The adoption of the model plant *Arabidopsis thaliana* has enabled us to isolate *mto* mutants that over-accumulate soluble methionine. Through the analysis of these *mto* mutants, valuable insight has been gained into the regulation and *in vivo* dynamics of the methionine biosynthetic pathway in higher plants. Each of the three mutant *mto* loci identified to date (*mto1*, *mto2*, *mto3*) disrupt different processes within the pathway and display distinct phenotypic profiles. They have also revealed that, in addition to the common feedback controls, the pathway is subject to changing temporal and spatial regulation over the course of the plant life cycle.

Key words: Aspartate family amino acids, developmental regulation, metabolic regulation, *S*-adenosylmethionine.

The amino acid methionine serves not only as a component of proteins, but is required for the initiation of protein synthesis and as the direct precursor of *S*-adenosylmethionine (SAM). SAM functions as the primary biological methyl donor in many transmethylation reactions, including that of proteins, phospholipids, nucleic acids and secondary metabolites (Chiang et al. 1996; Moffatt and Weretilnyk 2001), and is an important intermediate of polyamines, spermidine and spermine syntheses. In plant tissues, SAM is also metabolised into the phytohormone ethylene (Matthews 1999).

Methionine is one of the essential amino acids for non-ruminant animals including humans. It must therefore be obtained from the diet, of which plants serve as the ultimate source. Of significant concern is the fact that methionine is a limiting amino acid in several important agricultural crops such as soybean. In order to improve the methionine content in plants, a clear understanding of the regulation of methionine biosynthesis is required. This review aims to outline the knowledge gained through the analysis of genetic mutants affecting the methionine biosynthetic pathway, in addition to highlighting the new questions these studies have raised.

Overview of the methionine biosynthetic pathway

Methionine derives its carbon moiety from aspartic acid, a feature it shares in common with lysine, threonine and isoleucine, all of which are members of the aspartic acid family of amino acids. The biosynthesis of methionine is therefore part of a larger, complex pathway that involves the biosynthesis of other amino acids, and is best described as a component of aspartic acid metabolism subject to multiple regulatory controls (Figure 1).

Steps common to other aspartic acid family amino acids

The first two steps of the methionine biosynthetic pathway are common for all the aspartic acid family amino acids. Aspartic acid is first converted to aspartate phosphate by aspartate kinase (AK; EC 2.7.2.4), followed by the synthesis of aspartate semialdehyde. Like in many other first step enzymes of biosynthetic pathways, activity of AK isozymes is subject to feedback regulation by either threonine or lysine (Rognes et al. 1983; Heremans and Jacobs 1995; Azevedo et al. 1997). However, disruption of this regulation does not affect methionine accumulation (Shaul and Galili 1992; Heremans and Jacobs 1994), demonstrating that methionine biosynthesis is tightly controlled further

Abbreviations: CGS, cystathionine γ-synthase; OPH, *O*-phosphohomoserine; SAM, *S*-adenosylmethionine; SAMS, *S*-adenosylmethionine synthetase; SMM, *S*-methylmethionine; TS, threonine synthase.
This article can be found at http://www.jspcmb.jp/

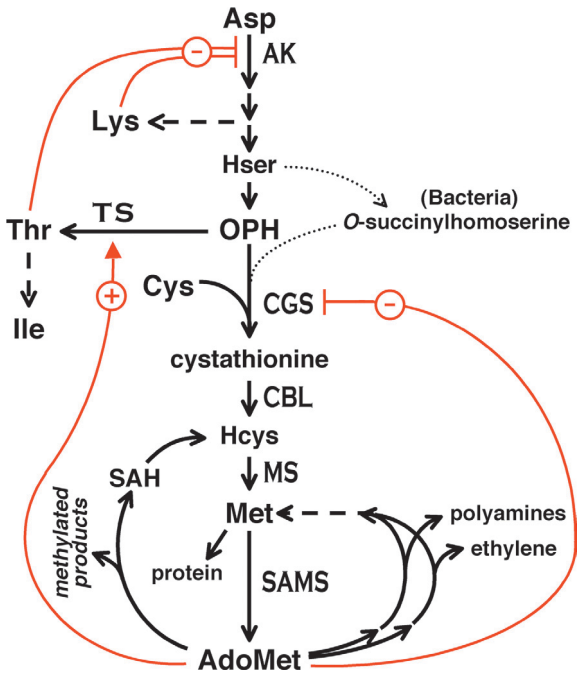

Figure 1. Outline of the biosynthetic and metabolic pathway for aspartic acid family amino acids and SAM in higher plants. Dashed lines indicate multiple reaction steps. Dotted lines indicate the branch point of threonine and methionine pathways in bacteria. Major regulatory steps are indicated with red lines with the "+" (positive feedback) and "−" (negative feedback) signs. AK, aspartate kinase; CBL, cystathionine β-lyase; Hcys, homocysteine; Hser, homoserine; MS, methionine synthase; SAH; *S*-adenosylhomocysteine.

downstream and that aspartic acid is not a limiting factor for the pathway.

Following the first branch point separating lysine biosynthesis from that of methionine, threonine and isoleucine, homoserine is converted to *O*-phosphohomoserine (OPH) by homoserine kinase (EC 2.7.1.39). OPH marks the second branch point in the pathway that divides methionine biosynthesis from that of threonine and isoleucine. Threonine synthase (TS; EC 4.2.99.2) and cystathionine γ-synthase (CGS; EC 2.5.1.48, formerly assigned as EC 4.2.99.9) both catalyse a nucleophilic attack on OPH. In the case of TS, a water molecule is used as the nucleophile resulting in threonine formation. On the other hand, CGS uses cysteine as the nucleophile to form cystathionine and marks both the entry of a sulfur atom into the pathway, and the first committed step of methionine biosynthesis.

Biochemical analyses of TS from several plant species have shown that it is not feedback-regulated by threonine or isoleucine, however, its activity did appear to be influenced by the methionine pathway. SAM, the direct metabolite of methionine, markedly stimulated the activity of plant TS enzymes in a reversible and co-operative manner (Madison and Thompson 1976; Aarnes 1978; Giovanelli et al. 1984). This regulation is unique to higher plants, as the bacterial enzyme is not stimulated in this manner (Curien et al. 1996).

Steps unique to methionine biosynthesis

The condensation of OPH and cysteine by CGS to form cystathionine represents the first step in the pathway that is unique to methionine biosynthesis. As such, it is expected that this step is important for regulating methionine accumulation (Ravanel et al. 1998b). Markedly reduced levels of extractable CGS activity were observed in both *Lemna* (Thompson et al. 1982b) and barley (Rognes et al. 1986) plants when grown in the presence of exogenous methionine, suggesting that methionine or one of its metabolites negatively regulates the amount of active CGS. However, activity of the CGS enzyme was not inhibited by methionine or SAM *in vitro*, indicating that CGS is not an allosteric enzyme (Thompson et al. 1982a), and therefore the mechanism of this regulation was not known. Treatment of barley plants with lysine and threonine, which inhibits the aspartic acid metabolic pathway and thus reduces methionine levels, also resulted in an almost four-fold increase in the levels of extractable CGS activity (Rognes et al. 1986).

Following the CGS reaction, methionine is synthesised in two additional steps that are catalysed by cystathionine β-lyase (EC 4.4.1.8; cystathionine→homocysteine) and methionine synthase (EC 2.1.1.14), respectively. Methionine synthase catalyses incorporation of a methyl group from methyltetrahydrofolate into homocysteine to form methionine. Methionine is either directly incorporated into protein or metabolised into SAM. The synthesis of SAM from methionine is catalysed by SAM synthetase (SAMS; EC 2.5.1.6) and the activity of this enzyme has been shown to be inhibited by SAM in pea seedlings (Aarnes 1977). The Arabidopsis genome contains at least three, and possibly four, genes for *SAMS* (Peleman et al. 1989b; Shen et al. 2002). Expression of *SAMS1* and *SAMS2* genes were compared with enzyme activity that indicated developmental regulation of these two genes occurs primarily at the mRNA level (Peleman et al. 1989a).

Recycling of methionine

SAM is used in a wide variety of biological reactions and represents a major pathway of methionine metabolism. The flux through methionine was analysed in *Lemna* and it was determined that over 80% of methionine is metabolised into SAM, of which approximately 90% is used for transmethylation reactions (Giovanelli et al. 1985). The product of these methylation reactions in higher plants is *S*-adenosylhomocysteine. *S*-Adenosylhomocysteine is recycled to homocysteine by adenosylhomocysteinase (EC 3.3.1.1), prior to the re-incorporation of a methyl group by methionine synthase and regeneration of the methionine molecule

(methionine cycle). Methionine synthase therefore represents both the last reaction of methionine biosynthesis and of methionine recycling following methylation reactions. SAM also serves as a precursor for biosynthesis of ethylene and polyamines. The secondary product of these pathways is 5′-methylthioadenosine, which is directly recycled back to methionine in four enzyme-catalysed reactions that conserves the sulfur and methyl group (5′-methylthioadenosine cycle). Despite the large flux of methionine through SAM, there is no net loss of methionine due to recycling, and thus protein synthesis is the only major reaction that consumes an entire methionine molecule.

An additional metabolic pathway involving the recycling of methionine has been shown to exist in higher plants and is referred to as the *S*-methylmethionine (SMM) cycle (Mudd and Datko 1990). SAM:methionine *S*-methyltransferase (EC 2.1.1.12) catalyses the formation of SMM from methionine in a reaction utilising SAM as a methyl donor (Giovanelli et al. 1980; Mudd and Datko 1990; James et al. 1995). Two methionine molecules are then regenerated from SMM by transfer of a methyl group from SMM to homocysteine. This second reaction is catalysed by homocysteine *S*-methytransferase (EC 2.1.1.10) (Giovanelli et al. 1980; Ranocha et al. 2000). SMM was identified as a major component in the phloem of several higher plant species (Bourgis et al. 1999), suggesting that it has a role in the transport of methionine in plant tissues. The first reaction of the SMM cycle may therefore serve to convert methionine into a more transportable form, where it is then reconverted back to methionine in the sink tissues. A model has been proposed where the SMM cycle was suggested to also have a major role in the homeostasis of SAM levels in higher plants (Ranocha et al. 2001).

Regulating metabolic flux to methionine in the pathway

As described above, early pioneering work identified many of the steps involved in the aspartic acid metabolic pathway. These studies had a strong biochemical basis and revealed much information about the enzymes involved and their activities. However, the regulatory mechanisms and *in vivo* dynamics of the methionine biosynthetic pathway remained unknown. Levels of the CGS enzyme, as opposed to enzymatic activity, were known to respond to changes in methionine availability, although the mechanism by which this occurred was unclear. Moreover, TS activity appeared to be influenced by the methionine metabolic product SAM, yet it was unknown whether the *in vivo* interactions between the threonine and methionine branch points had any influence on the regulation of methionine accumulation.

Significant advances in our understanding on the regulation of the methionine biosynthetic pathway in plants were provided through the isolation and characterisation of genetic mutants in which methionine accumulation is altered (Inaba et al. 1994; Chiba et al. 1999; Bartlem et al. 2000; Goto et al. 2002; Shen et al. 2002; Onouchi et al. 2005), as described in further detail below.

Genetic approaches using Arabidopsis

Ethionine is a toxic analogue of methionine (Alix 1982) that has been used to isolate methionine over-accumulating mutants of bacteria (Kappy and Metzenberg 1965) and yeast (Sorsoli et al. 1964; Mertz and Spence 1972). The phenotype of ethionine-resistance has also been used to successfully identify plant cell lines of tobacco, soybean, carrot and alfalfa that over-accumulate soluble methionine (Widholm 1976; Reish et al. 1981; Gonzales et al. 1984; Greenberg et al. 1988).

In recent times, Arabidopsis has been adopted as a genetic tool to further elucidate the regulation and *in vivo* dynamics of the methionine biosynthetic pathway in higher plants. The general approach has been to subject wild-type seeds to a mutagenesis treatment, and screen for mutants displaying a phenotype of methionine over-accumulation. This screening involves the germination of mutant seeds on sterile media containing a toxic concentration of ethionine. Toxicity of ethionine is explained as that it is used in many of the cellular reactions, including protein synthesis and SAM synthesis, in place of methionine. Mutant plants that over-accumulate soluble methionine are able to overcome the toxic levels of ethionine and survive, whereas wild-type plants die soon after germination (Figure 2). Once confirmed to contain elevated levels of soluble methionine, the mutant plants are referred to with the nomenclature *mto* (for methionine over-accumulation) mutants.

Soluble methionine levels in wild-type plants are generally low and require sensitive high performance liquid chromatography analysis. As a result, a screen for mutants with reduced methionine accumulation has yet to be developed, although severe growth defects following specific inhibition of the biosynthetic pathway suggest they may not survive (Datko and Mudd 1982; Thompson et al. 1982b).

To date, three *mto* loci have been identified that affect different steps of the biosynthetic pathway and have provided important insight into regulation of methionine accumulation in whole plants. The *mto1* (Inaba et al. 1994), *mto2* (Bartlem et al. 2000) and *mto3* (Goto et al. 2002; Shen et al. 2002) mutants contain lesions in genes encoding CGS, TS and SAMS, respectively.

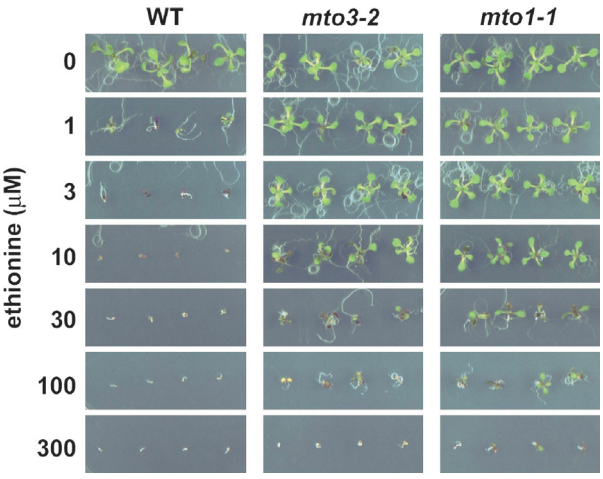

Figure 2. Ethionine resistance of *mto* mutants. Wild-type, *mto3-2* and *mto1-1* seeds were sown on hydroponic culture medium containing various concentrations of ethionine. Reprinted from Goto et al. (2002) with permission from the Genetic Society of Japan.

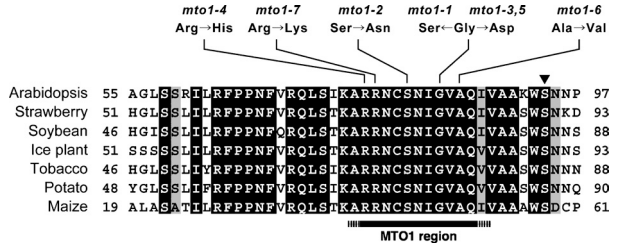

Figure 3. *mto1* mutation sites and the MTO1 region. *mto1* mutation sites are indicated above the amino acid sequence alignment of CGS from seven plant species for the highly conserved region. Identical amino acid residues are reversed and similar ones are shaded. The *MTO*1 region marked below the alignment was identified by alanine-substitution experiments (Ominato et al. 2002). Filled arrowhead indicates the position of translation arrest induced by SAM. Genbank/EMBL/DDBJ accession numbers are; Arabidopsis, AF039206; strawberry (*Fragaria vesca*), FVAJ1451; soybean (*Glycine max*), AF141602, BG363230, and 10 other ESTs; ice plant (*Mesembryanthemum crystallinum*), AF069317; tobacco (*Nicotiana tabacum*), AF097180, AB035300; potato (*Solanum tuberosum*), AF144102; maize (*Zea mays*), AF007785.

CGS enzyme is feedback regulated at the level of mRNA stability: *mto1* mutant

The first Arabidopsis methionine over-accumulating mutant to be isolated was *mto1-1* (Inaba et al. 1994). As suggested by its ethionine-resistant phenotype, soluble methionine levels in young *mto1-1* plants accumulated up to 40-fold higher than that in wild type. Surprisingly, accumulation of other aspartic acid family amino acids were not affected, demonstrating that the aspartic acid metabolic pathway is able to compensate for the increased metabolic flux to methionine without limiting the other branches of the pathway.

Genetic mapping and sequencing identified the causative mutation as a single base-pair change within the exon 1 coding region of the *CGS* gene (Chiba et al. 1999). Although the exon 1 amino acid sequence is generally poorly conserved, the *mto1* mutation resides within a 40-residue region that is highly conserved among plant species. All subsequent *mto1* alleles were also found to be located within this region. Alanine-substitution experiments identified a short stretch of amino acid sequence, Arg-Arg-Asn-Cys-Ser-Asn-Ile-Gly-Val-Ala-Gln, that covers the *mto1* mutation sites that is important for the regulation, which is now referred to as the MTO1 region (Ominato et al. 2002) (Figure 3). As a result of the mutation within the *CGS* exon 1 coding sequence, *CGS* mRNA levels were two- to four-fold higher in *mto1* than in wild-type plants, which is reflected by elevated levels of CGS protein and enzyme activity (Chiba et al. 1999; Ominato et al. 2002). Analysis of the regulation defect in *mto1* revealed that wild-type *CGS* is negatively regulated in response to exogenous application of methionine, and that post-transcriptional regulation at the level of mRNA stability plays a major role (Chiba et al. 1999). Additional studies have subsequently shown that the effector of this feedback regulation is not methionine itself, but rather its direct metabolite, SAM (Chiba et al. 2003).

The above work provided a mechanistic explanation for earlier findings that levels of extractable CGS were reduced by methionine treatment (Thompson et al. 1982b; Rognes et al. 1986). Moreover, it contributed to our understanding of the dynamics of the aspartic acid metabolic pathway by providing *in vivo* evidence that regulation of CGS levels is required for the strict control of methionine biosynthesis, and that compensatory mechanisms exist to adjust for changes in methionine accumulation and ensure that levels of other amino acids in the common pathway remain constant.

In addition to demonstrating the role of *CGS* regulation in controlling methionine accumulation, analysis of the *mto1* mutants has also led to the discovery of a novel feedback regulatory mechanism in plants (Onouchi et al. 2005). Although increased SAM levels result in *CGS* mRNA degradation, the nascent exon 1 peptide sequence is also required for this regulation, and thus translation of the mRNA must first be initiated (Chiba et al. 2003; Lambein et al. 2003). It has recently been shown that SAM actually first induces a temporal arrest in translation elongation immediately downstream of the MTO1 region, suggesting that the MTO1 region acts within the ribosomal exit tunnel, and that this translation arrest triggers degradation of its own mRNA (Onouchi et al. 2005) (Figure 4).

Threonine biosynthesis regulates metabolic flux to methionine: *mto2* mutant

Despite the findings that expression of the *CGS* gene is

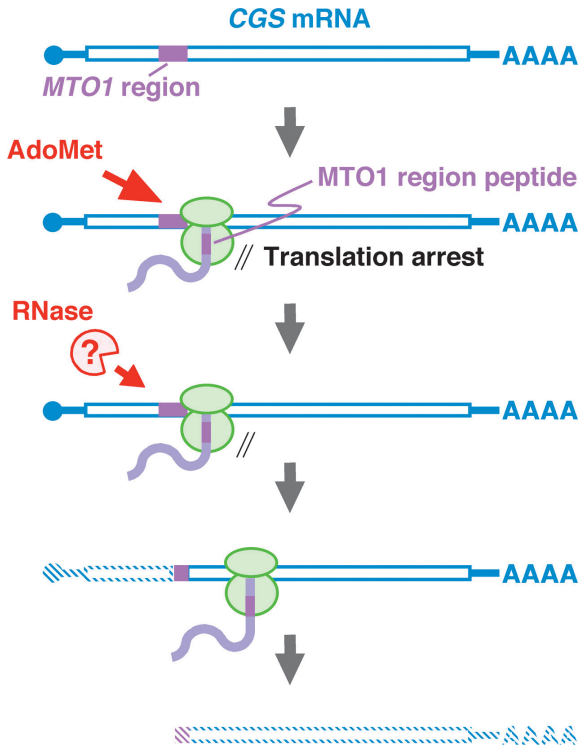

Figure 4. Model for the feedback regulation of *CGS* mRNA stability. When *CGS* mRNA is translated in the presence of SAM, temporal translation arrest occurs immediately downstream of the MTO1 region. This translation arrest triggers *CGS* mRNA degradation (Onouchi et al. 2005). A 5′-truncated RNA species are formed as degradation intermediate (Chiba et al. 1999; Chiba et al. 2003)

feedback-regulated, several reports indicated that regulation of the *CGS* gene alone is not sufficient to regulate methionine biosynthesis in higher plants and that other mechanisms are also involved (Thompson et al. 1982b, 1982a; Ravanel et al. 1998b). Based on determined K_m values and estimated subcellular concentration of its substrates in Arabidopsis, it has been proposed that the CGS reaction normally proceeds at approximately 1–2% of its maximal rate, and therefore, CGS is not the rate-limiting step in methionine biosynthesis (Ravanel et al. 1998a). Inhibitor studies of CGS in *Lemna* also revealed that only 16% of CGS control activity is necessary for normal rates of methionine biosynthesis and plant growth, and also that down-regulation of CGS to 15% of control activity is not sufficient to reduce the rate of methionine biosynthesis (Thompson et al. 1982b).

The methionine biosynthetic pathway in bacteria does not branch at OPH, but rather at an earlier step where homoserine is first converted to *O*-succinylhomoserine by homoserine *O*-transsuccinylase (EC 2.3.1.46), prior to a CGS-catalysed reaction resulting in cystathionine formation (Chattopadhyay et al. 1991) (Figure 1). The competition between TS and CGS for the common OPH substrate is therefore unique to higher plants. Studies in tobacco and soybean cell lines had suggested that OPH is preferentially drawn into threonine biosynthesis and that depression of TS allows increased methionine biosynthesis (Greenberg et al. 1988; Muhitch 1997). In addition, TS activity was shown to be stimulated by SAM *in vitro* (Madison and Thompson 1976; Aarnes 1978; Giovanelli et al. 1984). Although these studies indicated that the competition between TS and CGS may have a role in regulating methionine biosynthesis, no experimental evidence for this was available and the hypothesis assumed OPH availability was limited. Simple competition also did not explain the fact that threonine was not severely reduced in the *mto1* methionine over-accumulating mutant (Inaba et al. 1994).

The Arabidopsis *mto2-1* mutant was isolated in an independent screen from that of *mto1*, and over-accumulated soluble methionine in young rosettes to approximately 20-fold higher than that in wild type. Unlike that observed for *mto1*, soluble threonine levels were markedly reduced in young *mto2-1* rosettes to 6% of that in wild type, whereas accumulation of lysine and isoleucine was unaffected (Bartlem et al. 2000). The amino acid accumulation profile of *mto2-1* indicated that the branch point in the methionine biosynthetic pathway between methionine and threonine was affected. As threonine levels were normal in *mto1* mutants, it was unlikely that the defect in *mto2-1* caused increased activity of methionine-specific steps. A defect in SAM biosynthesis could explain the *mto2-1* phenotype, which would result in reduced activation of TS enzyme. However, SAM accumulation was three-fold higher in young *mto2-1* rosettes, suggesting this was not the case.

A single base-pair mutation within the gene encoding TS that altered the amino acid sequence of the enzyme active site was identified as the causative lesion in *mto2-1* (Bartlem et al. 2000). Moreover, the mutation was shown to reduce TS activity by functional complementation of an *E. coli thrC* auxotrophic mutant (Bartlem et al. 2000) (Figure 5A). Methionine over-accumulation in *mto2-1* was therefore the direct result of reduced threonine biosynthesis, revealing that TS and CGS do indeed compete for a limited substrate *in vivo* (Bartlem et al. 2000; Lee et al. 2005). Interestingly, although TS mRNA and protein levels were normal in *mto2-1*, accumulation of *CGS* mRNA and protein were negatively correlated with that of methionine and reduced to approximately 40% of that in wild type (Bartlem et al. 2000) (Figure 5B). The reduced levels of CGS associated with methionine over-accumulation showed that the regulatory mechanism controlling *CGS* mRNA accumulation in response to increased SAM (Chiba et al. 1999; Chiba et al. 2003) was functioning normally. Methionine was able to over-accumulate 20-fold in *mto2-1* when CGS levels were markedly reduced. This fact provides a clear *in vivo* demonstration that TS

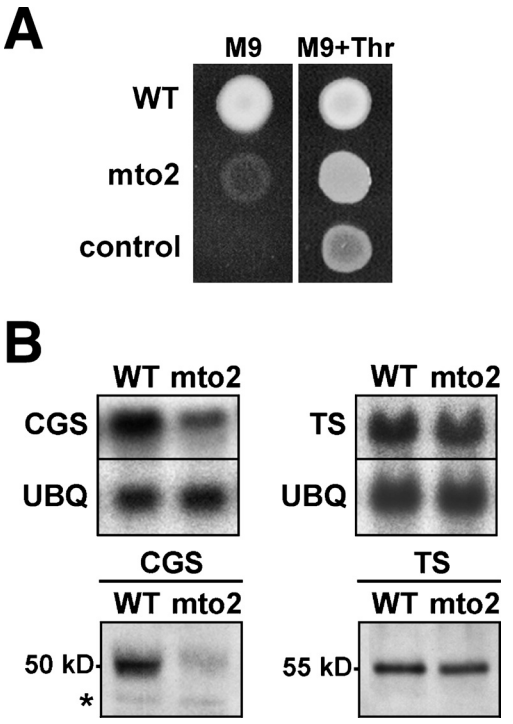

Figure 5. Role for TS activity in regulating methionine biosynthesis demonstrated by the *mto2-1* mutant. (A) Functional complementation of an *E. coli thrC* mutation that lacks TS activity. The *E. coli* mutant was transformed with wild-type Arabidopsis *TS* (WT), *mto2-1 TS* gene (mto2), or an empty vector (control). Growth on minimal medium (M9, left panel) shows that wild-type Arabidopsis *TS* is functional in *E. coli*, whereas *mto2-1 TS* gene encodes an enzyme with reduced activity. Right panel shows growths on minimal medium supplemented with threonine. (B) Accumulation of TS and CGS in *mto2-1*. *CGS* mRNA (upper panel) and protein (lower panel) levels are markedly reduced in *mto2-1*, however, methionine over-accumulates 20-fold revealing that TS activity is required for strict regulation of methionine biosynthesis and regulation of *CGS* gene alone is not sufficient. The band marked with an asterisk was also detected with the pre-immune serum. Reprinted from Bartlem et al. (2000) with permission from the American Society of Plant Biologists.

activity has a key role in regulating the carbon flow into the methionine biosynthetic pathway and, when TS activity is compromised, the regulation of CGS alone is not sufficient. This is in agreement with the finding that antisense repression of the *TS* gene in transgenic potato resulted in a reduction of TS activity to 6% of wild type and up to 230-fold increase in soluble methionine in leaf and tuber tissues, respectively (Zeh et al. 2001).

The *mto2-1* mutation also revealed another interesting aspect of the dynamics of aspartic acid metabolism: similar concentrations of isoleucine in *mto2-1* and wild type indicated that even when soluble threonine is reduced to 6% of that in wild type, this is sufficient to support isoleucine biosynthesis, and that threonine may even be preferentially drawn into isoleucine biosynthesis to fulfil this demand prior to being utilized for protein synthesis.

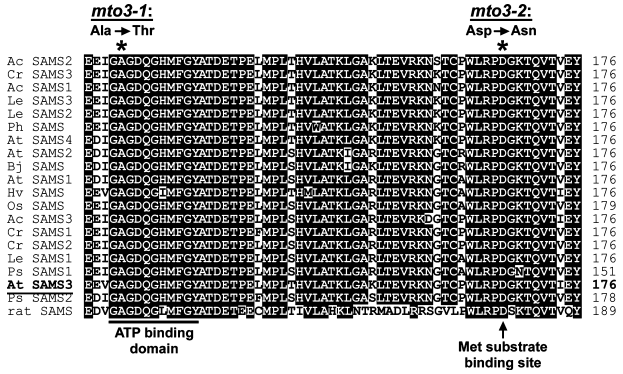

Figure 6. *mto3* mutations alter the SAMS active sites. Alignment of SAMS protein sequences from plants and rat showing high conservation of the active site and positions of the *mto3-1* and *mto3-2* mutations. Ac SAMS1, *Actinidia chinensis* SAMS (Genbank/EMBL/DDBJ accession number U17239); Ac SAMS2, *A. chinensis* SAMS (U17240); Ac SAMS3, *A. chinensis* SAMS (U17241); At SAMS1, *A. thaliana* SAMS1 (M55077); At SAMS2, *A. thaliana* SAMS2 (M33217); At SAMS3, *A. thaliana* SAMS3 (AY037214); At SAMS4, *A. thaliana* putative SAMS (AF367310); Bj SAMS, *Brassica juncea* SAMS (X80362); Cr SAMS1, *Catharanthus roseus* SAMS1 (Z71271); Cr SAMS2, *C. roseus* SAMS2 (Z71272); Cr SAMS3, *C. roseus* SAMS3 (Z71273); Hv SAMS, *Hordeum vulgare* SAMS (D63835); Le SAMS1, *Lycopersicon esculentum* SAMS1 (Z24741); Le SAMS2, *L. esculentum* SAMS2 (Z24742); Le SAMS3, *L. esculentum* SAMS3 (Z24743); Os SAMS, *Oryza sativa* SAMS (Z26867); Ph SAMS, *Petunia hybrida* SAMS (X82214); Ps SAMS1, *Pisum sativum* SAMS1 (X82076); Ps SAMS2, *P. sativum* SAMS2 (X82077); rat SAMS, *Rattus norvegicus* SAMS (X15734). Modified from Goto et al. (2002) with permission from the Genetic Society of Japan.

Conversion of methionine to SAM is required for biosynthetic regulation: *mto3* mutant

Two alleles of the *mto3* mutation have been isolated in independent studies and both carry single base-pair changes within the coding region for the active site of the SAMS3 enzyme. The *mto3-1* mutation alters the ATP binding site within the active site (Shen et al. 2002), whereas *mto3-2* changes the Asp-167 residue that likely binds the methionine substrate (Goto et al. 2002) (Figure 6). Although both *mto3-1* and *mto3-2* affect the SAMS3 active site, the severity of the phenotypes were different in the two mutations. In young *mto3-1* plants, concentrations of methionine were 244-fold higher than that in wild type and SAM levels were reduced by 35% (Shen et al. 2002). On the other hand, methionine concentrations were 20-fold higher in young plants of the weaker *mto3-2* allele (Goto et al. 2002) and SAM levels were not detectably different (D. Goto, M. Ogi, S. Naito, unpublished data) (Table 1).

Intriguingly, there was not a linear relationship between the increases in methionine accumulation observed in *mto3* mutants and decrease in SAM, particularly in the case of *mto3-2*. This may be due to the influence of recycling pathways or compartmentalisation

Table 1. Threonine, methionine and SAM concentrations in young rosette leaves of *mto* mutant plants. Fold-increases as compared to wild type are shown.

Mutant	Background	Threonine	Methionine	SAM
mto1-1	Col-0	0.88[1)]	40.[1)]	2.8[2)]
mto2-1[2)]	WS	0.06	22.	3.0
mto3-1[3)]	WS	1.4	244.	0.65
mto3-2	Col-0	1.1[4)]	18.[4)]	1.0[5)]

[1)] 20 day-old plants (Inaba et al. 1994). [2)] 15 day-old plants (Bartlem et al. 2000). Note that the SAM concentrations of wild-type and *mto*1-1 plants reported in Inaba et al. (1994) have been corrected in Bartlem et al. (2000). [3)] Plants before bolting (Shen et al. 2002). [4)] 15 day-old plants (Goto et al. 2002). [5)] 15 day-old plants (D. Goto, M. Ogi, S. Naito, unpublished data).

of SAM pools within individual cells (see below). As mentioned above, Arabidopsis contains at least three *SAMS* genes, and possibly a fourth (Shen et al. 2002). The fact that these other genes do not complement the *mto3* mutations demonstrates that there is little redundancy and that the individual *SAMS* genes have likely evolved distinct functions in methionine metabolism. Moreover, the two mutants were isolated in the *SAMS3* gene, and not in other *SAMS* genes, further suggesting that functional SAMS3 enzyme is specifically required for regulation of methionine accumulation. Mutations in *SAMS3* prevent flow of excess methionine into the metabolic pathway, however, they still allow normal flux to methionine at the OPH branch point. Combined with the finding that CGS levels at least in *mto3-2* are not appreciably affected, despite the 20-fold increase in methionine (Goto et al. 2002), this also suggested that SAM and not methionine is likely to be the effector of *CGS* regulation, which was later established by *in vitro* experiments (Chiba et al. 2003).

mto1, *mto2* and *mto3* represent three important genetic loci affecting different steps of the methionine biosynthetic pathway. Although they all over-accumulate soluble methionine, they each have distinct amino acid profiles. In *mto1*, methionine and SAM concentrations are increased, whereas threonine levels are normal. In contrast, increased methionine and SAM in *mto2* is associated with a severe reduction in threonine accumulation. *mto3* mutants have a more specific profile in that they only accumulate methionine, and SAM levels are either slightly reduced or not appreciably affected (Table 1).

Remaining questions about the dynamics of methionine biosynthesis

In order to fully understand the regulation of metabolic pathways in plants, it is important to remember that they are present within a multicellular, eukaryotic environment. As such, they are subject to temporal and spatial developmental regulation, in addition to possible compartmentalisation within individual cells. The methionine over-accumulating mutants have provided a greater understanding on the dynamics of carbon flux through the methionine biosynthetic pathway, however, they have also raised several new questions.

Does CGS also have a role in methionine metabolism?

The *mto1* mutant alleles all reside within a highly conserved region of the CGS exon 1 polypeptide that is involved in the feedback regulation of *CGS* mRNA stability by SAM. Unlike that observed in *Lemna* and Arabidopsis, analysis of antisense TS suppression lines in potato suggested that they lack the feedback regulation mechanism and rely primarily on TS activity for control of the methionine biosynthetic pathway (Zeh et al. 2001). This was surprising as the MTO1 region has also been conserved in the potato *CGS* gene (Figure 3), indicating it may also serve another function.

Additional studies in tobacco have raised the interesting possibility that the N-terminal region of CGS may be involved in more than just regulating *CGS* mRNA stability, but may also control methionine accumulation by somehow regulating the methionine metabolic pathway (Hacham et al. 2002). As expected, over-expression of wild-type Arabidopsis *CGS* in transgenic tobacco resulted in increased methionine accumulation. In contrast, over-expression of a *CGS* construct lacking the MTO1 region resulted in not only increased methionine but also high levels of ethylene and methionine catabolic products (Hacham et al. 2002).

Complexity introduced by compartmentalisation

The majority of the aspartic acid metabolic pathway occurs within the chloroplasts of plant cells (Wallsgrove et al. 1983; Ravanel et al. 1998b), although the final steps of methionine biosynthesis are unique in that chloroplasts only contain approximately 70% of the cystathionine β-lyase activity and essentially no methionine synthase activity (Wallsgrove et al. 1983). SAMS activity is thought to be present in the cytoplasm (Wallsgrove et al. 1983; Peleman et al. 1989b). The separation of SAM synthesis from that of CGS activity explains the requirement for a complex mechanism in which SAM induces the degradation of *CGS* mRNA during translation in the cytoplasm prior to transport of the protein to the chloroplast. This also highlights the strong possibility of additional physical separation of regulatory components within the biosynthetic pathway. For example, it is interesting to know why no reduction in SAM was seen in *mto3-2*, although the increase in methionine was assumed to be due to decreased metabolism of methionine into SAM.

It has recently been shown that one of the methionine synthase isoforms in Arabidopsis is localised to the

chloroplast, suggesting that chloroplasts are autonomous for *de novo* methionine biosynthesis and that the other two isoforms in the cytoplasm are more likely to be involved in methionine synthesis through recycling pathways (Ravanel et al. 2004). Moreover, it was also found that SAM is transported into the chloroplasts in a carrier-mediated process, raising the possibility of different subcellular concentrations (Ravanel et al. 2004). It is tempting to speculate that similar functional differences exist for the SAMS enzymes. Although evidence is lacking, one possible explanation for the lack of correlation between decrease of SAM and increase of methionine in *mto3* mutants is that the recycling pathway is specifically disrupted, introducing a rate-limiting step on the cycling of methionine to SAM. Under this model, *de novo* synthesis of methionine and SAM would not be affected and may even be regulated to maintain required concentrations of SAM. Isolation of mutants in the other *SAMS* genes, their phenotypic comparisons and double-mutant analysis is likely to provide further insight into the functional differences.

The methionine biosynthetic pathway is subject to additional developmental regulation

The over-accumulation of methionine has been found to vary significantly in the *mto* mutants depending on the age of the plants and the tissue examined. In *mto1* mutants, methionine levels are up to 40-fold higher than wild type in leaves of young plants, however, this over-accumulation decreases after 25 days and by 40 days the methionine level is similar to that in wild type (Inaba et al. 1994; Bartlem et al. 2000; Goto et al. 2002). In the *mto2* mutant, threonine levels also increased from 6% of wild type in young leaves, up to 85% of wild type levels in 30-day-old plants (Bartlem et al. 2000) (Figure 7). In fact, *mto2* seedlings require threonine supplementation for normal growth when plated on synthetic agar medium. Despite the differences in their relative concentrations at specific stages, temporal patterns of threonine levels were similar between wild type and *mto2* in that several-fold increases were seen after 20 days of growth. In wild-type rosettes, this increase occurred without a reduction in soluble methionine concentration. It is interesting that the changes in methionine and threonine in both wild type and *mto2* occur as the plants enter the reproductive growth stage.

In older plants (40 days old), the concentration of soluble threonine was reduced in all tissues of *mto2* compared to that in wild type (Bartlem et al. 2000) (Figure 7). On the other hand, soluble methionine concentrations were similar to that in wild type, in contrast to that in young plants. This suggests that methionine biosynthesis may be under additional stricter control in ageing plants. The fact that reduced methionine over-accumulation was observed in all three

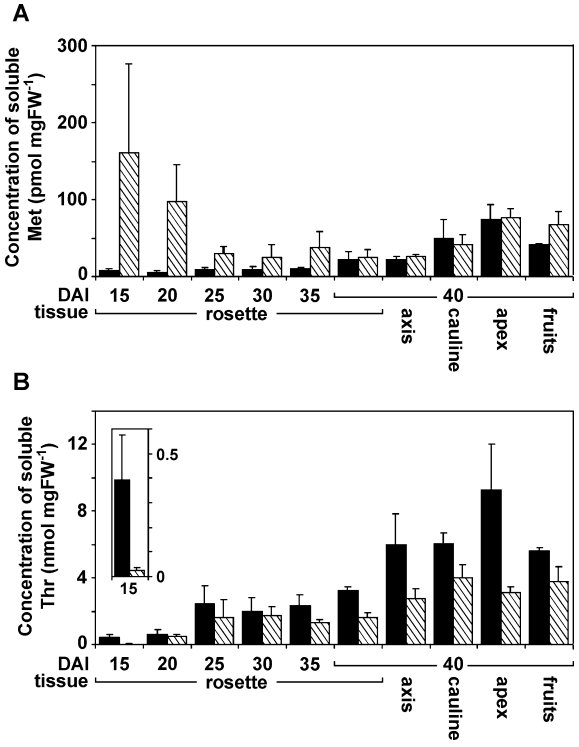

Figure 7. Developmental changes in methionine (A) and threonine (B) concentrations in wild-type (solid bars) and *mto2* mutant (hatched bars) plants. DAI, days after imbibition. Reprinted from Bartlem et al. (2000) with permission from the American Society of Plant Biologists.

mto mutants, which all affect different regulatory steps of the pathway, suggests that tissue-specific accumulation of methionine may be regulated through a translocation process. This is in agreement with several reports showing that excess methionine may be transported from source leaf tissue to reproductive sink tissues in older plants (Inaba et al. 1994; Naito et al. 1994; Kim et al. 2002).

Developmental regulation of methionine in *mto2* does, however, suggest that temporal reduction in methionine may not be simply due to translocation to sink tissues. Parallel increases of threonine in old leaves indicate relaxing of the competitive pressure on the OPH branch point following transition to reproductive growth (Bartlem et al. 2000). Down-regulation of CGS was also not responsible for the reduced competitive pressure, as *CGS* mRNA levels remained constant. It is apparent that the aspartic acid metabolic pathway and methionine biosynthesis is subject to complex regulatory mechanisms and that the dynamics of this regulation change over the course of the plant life cycle.

Concluding remarks

The genetic studies described above and others using transgenic plants (for reviews, see also Amir et al. 2002; Hesse et al. 2004) have begun to provide valuable insight

into the dynamics of methionine biosynthetic pathway in plants. It must be remembered that methionine biosynthesis is not a simple pathway with defined beginning and end points, but rather part of a complex metabolic pathway that involves synthesis of other amino acids and includes multiple metabolic fates and recycling events. In addition, these processes are occurring in different compartments within individual cells and are subject to complex spatial and temporal developmental regulation. It is clear that there is still much to be learnt and that this field will benefit from metabolomic analysis, continued biochemical studies and expansion of genetic studies to incorporate developmental aspects of the pathway.

Acknowledgements

The studies summarized in this article were supported by Research for the Future Program (97L00601) and Grants-in-Aid for Scientific Research (07454214, 09440262, 1144023 and 13440233) from the Japanese Society for the Promotion of Science, and Grants-in-Aid for Scientific Research on Priority Areas (06278102, 12138201 and 14035208) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

Aarnes H (1977) Partial purification and characterization of methionine adenosyltransferase from pea seedlings. *Plant Sci Lett* 10: 381–390

Aarnes H (1978) Regulation of threonine biosynthesis in barley seedlings (*Hordeum vulgare* L.). *Planta* 140: 185–192

Alix J (1982) Molecular aspects of the *in vivo* and *in vitro* effects of ethionine, an analog of methionine. *Microbiol Rev* 46: 281–295

Amir R, Hacham Y, Galili G (2002) Cystathionine γ-synthase and threonine synthase operate in concert to regulate carbon flow towards methionine in plants. *Trends Plant Sci* 7: 153–156

Azevedo RA, Blackwell RD, Smith RJ, Lea PJ (1997) Three aspartate kinase isoenzymes from maize. *Phytochemistry* 46: 395–419

Bartlem D, Lambein I, Okamoto T, Itaya A, Uda Y, Kijima F, Tamaki Y, Nambara E, Naito S (2000) Mutation in the threonine synthase gene results in an over-accumulation of soluble methionine in Arabidopsis. *Plant Physiol* 123: 101–110

Bourgis F, Roje S, Nuccio ML, Fisher DB, Tarczynski MC, Li CJ, Herschbach C, Rennenberg H, Pimenta MJ, Shen TL, Gage DA, Hanson AD (1999) *S*-methylmethionine plays a major role in phloem sulfur transport and is synthesized by a novel type of methyltransferase. *Plant Cell* 11: 1485–1497

Chattopadhyay MK, Ghosh AK, Sengupta S (1991) Control of methionine biosynthesis in *Escherichia coli* K12: A closer study with analogue-resistant mutant. *J Gen Microbiol* 137: 685–691

Chiang PK, Gordon RK, Tal J, Zeng GC, Doctor BP, Pardhasaradhi K, McCann PP (1996) *S*-Adenosylmethionine and methylation. *FASEB J* 10: 471–480

Chiba Y, Ishikawa M, Kijima F, Tyson RH, Kim J, Yamamoto A, Nambara E, Leustek T, Wallsgrove RM, Naito S (1999) Evidence for autoregulation of cystathionine γ-synthase mRNA stability in *Arabidopsis*. *Science* 286: 1371–1374

Chiba Y, Sakurai R, Yoshino M, Ominato K, Ishikawa M, Onouchi H, Naito S (2003) *S*-adenosyl-L-methionine is an effector in the posttranscriptional autoregulation of the cystathionine γ-synthase gene in *Arabidopsis*. *Proc Natl Acad Sci USA* 100: 10225–10230

Curien G, Dumas R, Ravanel S, Douce R (1996) Characterization of an *Arabidopsis thaliana* cDNA encoding an *S*-adenosylmethionine-sensitive threonine synthase—Threonine synthase from higher plants. *FEBS Lett* 390: 85–90

Datko AH, Mudd SH (1982) Methionine biosynthesis in *Lemna*: Inhibitor studies. *Plant Physiol* 69: 1070–1076

Giovanelli J, Mudd SH, Datko AH (1980) Sulfur amino acids in plants. In: Miflin BJ (ed) *The Biochemistry of Plants*. Academic Press, New York, pp 453–505

Giovanelli J, Mudd SH, Datko AH (1985) Quantitative analysis of pathways of methionine metabolism and their regulation in *Lemna*. *Plant Physiology* 78: 555–560

Giovanelli J, Veluthambi K, Thompson GA, Mudd SH, Datko AH (1984) Threonine synthase of *Lemna paucicostata* Hegelm. 6746. *Plant Physiol* 76: 285–292

Gonzales RA, Das PK, Widholm JM (1984) Characterization of cultured tobacco cell lines resistant to ethionine, a methionine analog. *Plant Physiol* 74: 640–644

Goto DB, Ogi M, Kijima F, Kumagai T, van Werven F, Onouchi H, Naito S (2002) A single-nucleotide mutation in a gene encoding *S*-adenosylmethionine synthetase is associated with methionine over-accumulation phenotype in *Arabidopsis thaliana*. *Genes Genet Syst* 77: 89–95

Greenberg JM, Thompson JF, Madison JT (1988) Homoserine kinase and threonine synthase in methionine-overproducing soybean tissue cultures. *Plant Cell Rep* 7: 477–480

Hacham Y, Avraham T, Amir R (2002) The N-terminal region of Arabidopsis cystathionine γ-synthase plays an important regulatory role in methionine metabolism. *Plant Physiol* 128: 454–462

Heremans B, Jacobs M (1994) Selection of *Arabidopsis thaliana* (L.) Heynh. mutants resistant to aspartate-derived amino acids and analogues. *Plant Sci* 101: 151–162

Heremans B, Jacobs M (1995) Threonine accumulation in a mutant of *Arabidopsis thaliana* (L.) Heynh. with an altered aspartate kinase. *J Plant Physiol* 146: 249–257

Hesse H, Kreft O, Maimann S, Zeh M, Hoefgen R (2004) Current understanding of the regulation of methionine biosynthesis in plants. *J Exp Bot* 55: 1799–1808

Inaba K, Fujiwara T, Hayashi H, Chino M, Komeda Y, Naito S (1994) Isolation of an *Arabidopsis thaliana* mutant, *mto1*, that overaccumulates soluble methionine: temporal and spatial patterns of soluble methionine accumulation. *Plant Physiol* 104: 881–887

James F, Nolte KD, Hanson AD (1995) Purification and properties of *S*-adenosyl-L-methionine: L-methionine *S*-methyltransferase from *Wollastonia biflora* leaves. *J Biol Chem* 270: 22343–22350

Kappy MS, Metzenberg RL (1965) Studies on the basis of ethionine-resistance in *Neurospora*. *Biochim Biophys Acta* 107: 425–433

Kim J, Lee M, Chalam R, Martin MN, Leustek T, Boerjan W (2002) Constitutive overexpression of cystathionine γ-synthase in *Arabidopsis* leads to accumulation of soluble methionine and *S*-methylmethionine. *Plant Physiol* 128: 95–107

Lambein I, Chiba Y, Onouchi H, Naito S (2003) Decay kinetics

of autogenously regulated *CGS1* mRNA that codes for cystathionine γ-synthase in *Arabidopsis thaliana*. *Plant Cell Physiol* 44: 893–900

Lee M, Martin MN, Hudson AO, Lee J, Muhitch MJ, Leustek T (2005) Methionine and threonine synthesis are limited by homoserine availability and not the activity of homoserine kinase in *Arabidopsis thaliana*. *Plant J* 41: 685–696

Madison JT, Thompson JF (1976) Threonine synthetase from higher plants: Stimulation by *S*-adenosylmethionine and inhibition by cysteine. *Biochem Biophys Res Commun* 71: 684–691

Matthews BF (1999) Lysine, threonine and methionine biosynthesis. In: Singh BK (ed) *Plant Amino Acids*: *Biochemistry and Biotechnology*. Marcel Dekker, New York, pp 205–225

Mertz JE, Spence KD (1972) Methionine adenosyltransferase and ethionine resistance in *Saccharomyces cerevisiae*. *J Bacteriol* 111: 778–783

Moffatt BA, Weretilnyk EA (2001) Sustaining *S*-adenosyl-L-methionine-dependent methyltransferase activity in plant cells. *Physiol Plant* 113: 435–442

Mudd SH, Datko AH (1990) The *S*-methylmethionine cycle in *Lemna paucicostata*. *Plant Physiol* 93: 623–630

Muhitch MJ (1997) Effects of expressing *E. coli* threonine synthase in tobacco (*Nicotiana tabacum* L.) suspension culture cells on free amino acid levels, aspartate pathway enzyme activities and uptake of aspartate into the cells. *J Plant Physiol* 150: 16–22

Naito S, Inaba-Higano K, Kumagai T, Kanno T, Nambara E, Fujiwara T, Chino M, Komeda Y (1994) Maternal effects of *mto1* mutation, that causes overaccumulation of soluble methionine, on the expression of a soybean β-conglycinin gene promoter-GUS fusion in transgenic *Arabidopsis thaliana*. *Plant Cell Physiol* 35: 1057–1063

Ominato K, Akita H, Suzuki A, Kijima F, Yoshino T, Yoshino M, Chiba Y, Onouchi H, Naito S (2002) Identification of a short highly conserved amino acid sequence as the functional region required for posttranscriptional autoregulation of the cystathionine γ-synthase gene in *Arabidopsis*. *J Biol Chem* 277: 36380–36386

Onouchi H, Nagami Y, Haraguchi Y, Nakamoto M, Nishimura Y, Sakurai R, Nagao N, Kawasaki D, Kadokura Y, Naito S (2005) Nascent peptide-mediated translation elongation arrest coupled with mRNA degradation in the *CGS1* gene of *Arabidopsis*. *Genes Dev* 19: 1799–1810

Peleman J, Boerjan W, Engler G, Seurinck J, Botterman J, Alliotte T, Van Montagu M, Inze D (1989a) Strong cellular preference in the expression of a housekeeping gene of *Arabidopsis thaliana* encoding *S*-adenosylmethionine synthetase. *Plant Cell* 1: 81–93

Peleman J, Saito K, Cottyn B, Engler G, Seurinck J, Van Montagu M, Inze D (1989b) Structure and expression analyses of the *S*-adenosylmethionine synthetase gene family in *Arabidopsis thaliana*. *Gene* 84: 359–369

Ranocha P, Bourgis F, Ziemak MJ, Rhodes D, Gage DA, Hanson AD (2000) Characterization and functional expression of cDNAs encoding methionine-sensitive and -insensitive homocysteine *S*-methyltransferases from Arabidopsis. *J Biol Chem* 275: 15962–15968

Ranocha P, McNeil SD, Ziemak MJ, Li CJ, Tarczynski MC, Hanson AD (2001) The *S*-methylmethionine cycle in angiosperms: ubiquity, antiquity and activity. *Plant J* 25: 575–584

Ravanel S, Block MA, Rippert P, Jabrin S, Curien G, Rebeille F, Douce R (2004) Methionine metabolism in plants—Chloroplasts are autonomous for *de novo* methionine synthesis and can import *S*-adenosylmethionine from the cytosol. *J Biol Chem* 279: 22548–22557

Ravanel S, Gakiere B, Job D, Douce R (1998a) Cystathionine γ-synthase from *Arabidopsis thaliana*: Purification and biochemical characterisation of the recombinant enzyme overexpressed in *Escherichia coli*. *Biochem J* 331: 639–648

Ravanel S, Gakiere B, Job D, Douce R (1998b) The specific features of methionine biosynthesis and metabolism in plants. *Proc Natl Acad Sci USA* 95: 7805–7812

Reish B, Duke SH, Bingham ET (1981) Selection and characterization of ethionine-resistant alfalfa (*Medicago sativa* L.) cell lines. *Theor Appl Genet* 59: 89–94

Rognes SE, Bright SWJ, Miflin BJ (1983) Feedback-insensitive aspartate kinase isoenzymes in barley mutants resistant to lysine plus threonine. *Planta* 157: 32–38

Rognes SE, Wallsgrove RM, Kueh JSH, Bright SWJ (1986) Effects of exogenous amino acids on growth and activity of four aspartate pathway enzymes in barley. *Plant Sci* 43: 45–50

Shaul O, Galili G (1992) Threonine overproduction in transgenic tobacco plants expressing a mutant desensitized aspartate kinase of *Escherichia coli*. *Plant Physiol* 100: 1157–1163

Shen B, Changjiang L, Tarczynski MC (2002) High free-methionine and decreased lignin content result from a mutation in the *Arabidopsis S*-adenosyl-L-methionine synthetase 3 gene. *Plant J* 29: 371–380

Sorsoli WA, Spence KD, Parks LW (1964) Amino acid accumulation in ethionine-resistant *Saccharomyces cerevisiae*. *J Bacteriol* 88: 20–24

Thompson GA, Datko AH, Mudd SH (1982a) Methionine synthesis in *Lemna*: Inhibition of cystathionine γ-synthase by propargylglycine. *Plant Physiol* 70: 1347–1352

Thompson GA, Datko AH, Mudd SH (1982b) Methionine synthesis in *Lemna*: Studies on the regulation of cystathionine γ-synthase, *O*-phoshohomoserine sulfhydrylase, and *O*-acetylserine sulfhydrylase. *Plant Physiol* 69: 1077–1083

Wallsgrove RM, Lea PJ, Miflin BJ (1983) Intracellular localization of aspartate kinase and the enzymes of threonine and methionine biosynthesis in green leaves. *Plant Physiol* 71: 780–784

Widholm JM (1976) Selection and characterization of cultured carrot and tobacco cells resistant to lysine, methionine, and proline analogs. *Can J Bot* 54: 1523–1529

Zeh M, Casazza AP, Kreft O, Roessner U, Bieberich K, Willmitzer L, Hoefgen R, Hesse H (2001) Antisense inhibition of threonine synthase leads to high methionine content in transgenic potato plants. *Plant Physiol* 127: 792–802