Chapter 5

Metabolism of Methionine in Plants and Phototrophic Bacteria

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Summary

The sulfur-containing amino acid methionine is a nutritionally important essential amino acid and the precursor of several metabolites that regulate plant growth and responses to the environment. New genetic and molecular data suggest that methionine synthesis and catabolism are coordinately regulated by novel post-transcriptional and post-translational mechanisms. This review focuses on new features reported for the molecular and biochemical aspects of methionine biosynthesis in higher plants with special emphasis on a comparison of the methionine biosynthetic pathway of plants with pathways of phototrophic bacteria (cyanobacteria). Particularly, the impact of the compartmentalization of methionine biosynthesis will be addressed with respect to regulatory aspects.

I. Introduction

Studying the regulation of methionine (Met) homeostasis is crucial for our understanding of physiological and biochemical processes in organisms. As organisms are exposed to environmental changes they have developed response mechanisms to keep metabolites in equilibrium. The process, termed adaptation, reflects the molecular/genetic and biochemical plasticity of a system. Compared to plants cyanobacteria are one of the oldest groups of photoautotrophic organisms on Earth (Schopf, 2000). They have colonized almost every available niche for the last 3.5 billion years, demonstrating an outstanding ability for adaptation to extremely different habitats and play a dominant role in the global nitrogen and carbon cycles. Thus, it was even not surprising to discover abundant cyanobacteria (Johnson and Sieburth, 1979) such as *Synechococcus* (Waterbury et al., 1979) and *Prochlorococcus*

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(Chisholm et al., 1988) thriving in environments with a very poor nutrient supply, such as the vast intertropical gyres of the oceans (Capone, 2000; Karl, 2002; Zehr and Ward, 2002; Delong and Karl, 2005), which were previously considered to be almost empty of living cells. However, their ecological importance was truly unexpected, since it is currently accepted that about half of the global primary production occurs in the oceans (Whitman et al., 1998) and that marine cyanobacteria contribute two-thirds of it (Goericke and Welschmeyer, 1993; Liu et al., 1997). Their metabolism is based on oxygenic photosynthesis, similar to that of eukaryotic algae and plants actually being related to the precursor of plant chloroplasts according to the endosymbiont theory. This process provides ATP and reducing equivalents from the splitting of water, which enables the bacteria to assimilate simple inorganic nutrients for their anabolic demands. Since the discovery of, e.g., *Prochlorococcus* in 1988 (Chisholm et al., 1988), its major importance in the ecology of the oceans has become evident, leading to a large number of genetic studies resulting in sequencing of the genomes of five representative *Prochlorococcus* ecotypes, MED4, MIT9313, MIT9312, and NATL2A (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi) and SS120 (http://www.sb-roscoff.fr/Phyto/ProSS120/), making *Prochlorococcus* one of the most-studied microorganisms from a genomic point of view (Dufresne et al., 2001; Rocap et al., 2003). However, since 2003 the genome of *Synechococ cus* is also sequenced (Palenik et al., 2003). The Kazusa Institute in Japan publicly provides sequence information of several cyanobacteria (www.kazusa.or.jp/cyano/). The striking ecological success of cyanobacteria has been the subject of many studies, focused on some of the most intriguing abilities of this organism to cope with the natural gradients of different parameters occurring along the water column, including light irradiance, which decreases almost four orders of magnitude from the ocean surface to the end of the euphotic zone. Due to their abundance and high metabolic activities, microorganisms may deplete the environment of essential nutrients. A prominent example of an environmental effect caused by the metabolic activity of cyanobacteria is the depletion of carbon dioxide from the atmosphere during the course of evolution, and the concomitant accumulation of the 'waste product' of photosynthesis, oxygen. Deprivation of essential nutrients is frequently the limiting factor in cyanobacterial cell growth, and the need to adapt to periods of nutrient limitation is a major source of selective pressure in diverse natural environments. Research in the past decade has led to fundamental new insights into the molecular mechanisms of these responses. Classically, acclimation responses to nutrient limitation are grouped into specific and general, or common, responses. The specific responses are the acclimation processes that occur as a result of limitation for a particular nutrient, whereas the general responses occur under any starvation condition. Cyanobacterial acclimation to limitation of the macronutrients phosphorus and nitrogen was studied in the past and summarized by Schwarz and Forchhammer (2005). As outlined by the authors little substantial progress has been made in understanding the molecular mechanisms underlying responses to sulfur limitation. This also applies to approaches investigating Met biosynthesis in cyanobacteria.

Met is an essential cellular constituent, an initiator of protein synthesis and a key player in many metabolic activities. Furthermore, Met is the precursor of SAM—S-adenosylmethionine—that is involved in many methylation reactions such as DNA and phospholipid methylation and synthesis of cyclopropane fatty acids. SAM also donates aminopropyl groups to diamines in the synthesis of polyamines such as spermidine, which neutralize the negative charge of nucleic acids in the cells (for reviews see Chiang et al., 1996; Fontecave et al., 2004), is precursor of the vitamin biotin, as well as to synthesize the 'aging' hormone ethylene responsible for fruit ripening. Furthermore, in algae and some bacteria Met is a source of atmospheric sulfur: dimethylsulphide (Amir et al., 2002; see

Abbreviations: CDMS – cobalamin-dependent Met synthase; CBL – cystathionine beta-lyase; CBS – cystathionine b-synthase; CGL – cystathionine g-lyase; CGS – cystathionine gamma-synthase; CIMS – cobalamin-independent Met synthase; HCys – homocysteine; HMT – HCys S-methyltransferase; HS – homocysteine synthase; HSK – homoserine kinase; HTA – homoserine transacetylase; HTS – homoserine trans-succinylase; Met, methionine; MMT – Met S-methyltransferase; MS – methionine synthase; MTA – methylthioadenosine; MTR – 5´-methylthioribose; MTR1P – 5´-methyl-thioribose 1-phosphate; OAHS – O-acetyl homoserine sulfhydrylase; OPHS – O-phosphohomoserine; OSHS – O-succinyl homoserine sulfhydrylase; Thr, threonine; SAH – S-adenosylhomocysteine; SAM – S-adenosylmethionine; SAMS – SAM synthetase; SMM – S-methylmethionine; SRH – S-ribosylhomocysteine;

also Chapters 14, 16 and 22, this issue). A derivative of Met, S-methylmethionine (SMM), is used as a major transport molecule for reduced sulfur in some plant species, connecting sink and source organs (Bourgis et al., 1999). Furthermore, Met is an essential amino acid required in the diet of non-ruminant animals. Human and monogastric mammals can synthesize only half of the 20 major proteinogenic amino acids and, therefore, must obtain the others from their diets. Major crops, such as cereals (e.g., corn, rice, wheat, etc.) and legumes, are low in Met (Tabe and Higgins, 1998; Hesse et al., 2001; Galili et al., 2005). Improved nutritional quality may help to solve problems encountered in cases where plant foods are the major or sole source of protein, such as in many developing countries, as well as plant feeds for livestock which are subsequently used as human food.

Recent findings indicate that Met intermediates such as homoserine has yet another important physiological role in microorganisms—it is involved in the synthesis of N-acyl homoserine lactone autoinducer molecules, which constitute quorum-sensing signals and act as cell density-dependent regulators of gene expression (Hanzelka and Greenberg, 1996; Val and Cronan, 1998; Yang et al., 2005). As a process, Met biosynthesis is widely distributed among the three domains of life (bacteria, archaea and eukarya) and could predate their divergence. However, the constituent steps in the pathway, the enzymes catalyzing these steps and the genes encoding these enzymes are not the same among all organisms and there is significant evolutionary plasticity at each of these levels. In plants and microorganisms Met is in general synthesized through three consecutive reactions catalyzed by cystathionine γ-synthase (CGS), cystathionine β-lyase (CBL), and Met synthase (MS) (Fig. 1). Met receives its carbon skeleton from the aspartate-family pathway, while its sulfur moiety is derived from cysteine (Cys). Eventually about 20% of the Met in plants at least is incorporated into proteins while 80% are converted to S-adenosylmethionine (SAM) which thus comprises the actual end product of this biosynthetic pathway as free Met does only occur in marginal concentrations in plants (Giovanelli et al., 1985). This fact indicates the high consumption of Met in different cellular processes. Furthermore, organisms evolved a Met salvage pathway to recover Met without de novo synthesis of the respective precursors. In

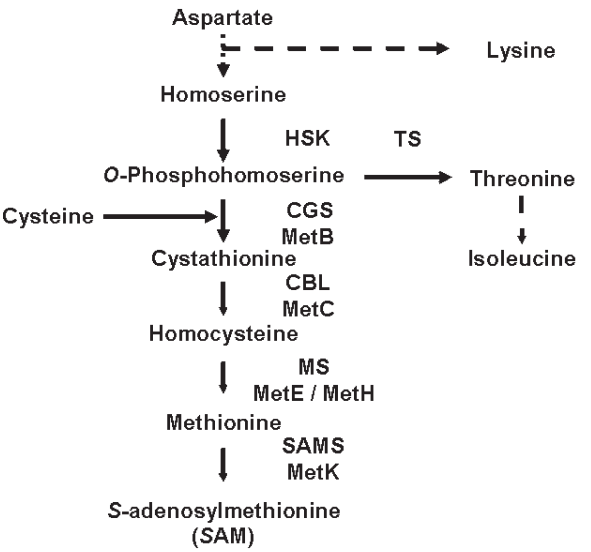

Fig. 1. Met focused biosynthetic pathway of amino acids of the aspartate family in plants. CGS, cystathionine γ-synthase; CBL, cystathionine β-lyase; MS, Met synthase; SAMS, S-AdoMet synthethase. Dashed arrows indicate multiple steps in lysine and isoleucine formation. Corresponding genes in *E. coli* are given in conventional nomenclature (met…).

plants, CGS, localized in chloroplasts, catalyses the formation of the thioether cystathionine from the substrates Cys and O-phosphohomoserine (OPHS) thus, connecting the aspartate-derived pathway to sulfur assimilation. In yeast, Met is synthesized by direct sulfhydration of O-acetylhomoserine and in bacteria in a different pathway with succinylhomoserine as substrate. It has thus to be emphasized that in microorganisms homoserine is the branch point intermediate leading to the synthesis of Met and threonine (Thr), whereas in plants OPHS is the last common intermediate. This difference of the branch point of the Met and Thr biosynthetic pathway in plants asks for an effective regulation of the respective enzymatic activities. Cyanobacterial orthologs of plant genes have been identified from the Genome Database for Cyanobacteria. Among the listed mutants defective in different pathways none of them is auxotrophic for Met. Genes for the biosynthetic as well as for the salvage pathway can be identified in this database. However, depending on the organism differences in gene annotation are evident and lined out in Gophna et al. (2005). The false annotation has severe consequences for predicting pathways enzymes. As long as the biochemical properties of

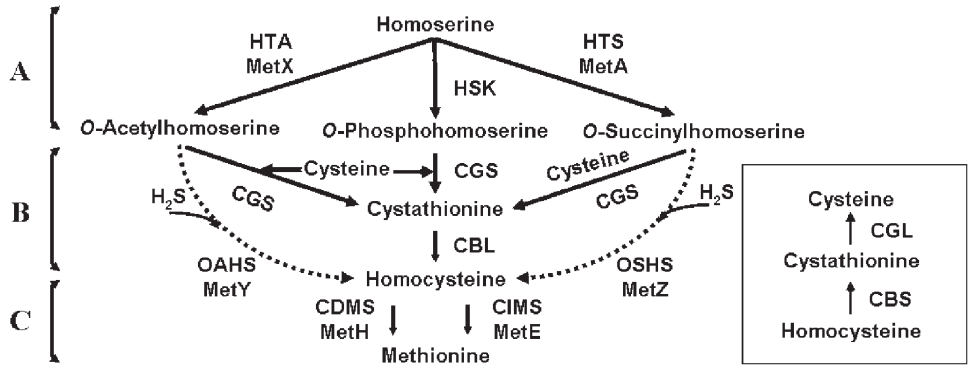

Fig. 2. The biochemical pathway of Met synthesis. Schematic presentation compares mechanistic differences in the synthesis of HCys in plants, bacteria, and fungi. HTA, homoserine trans-acetylase; HSK, homoserine kinase; HTS, homoserine trans-succinylase; CGS, cystathionine γ-synthase; CBL, cystathionine β-lyase; OAHS, O-acetyl homoserine sulfhydrylase (in fungi metY); OSHS, O-succinyl homoserine sulfhydrylase (in bacteria metZ); CDMS, cobalamin-dependent Met synthase (metH); CIMS, cobalamin-independent Met synthase (metE); CBS, cystathionine β-synthase; CGL, cystathionine γ-lyase. A, homoserine activation, B, sulfur incorporation, C, methylation. Dashed lines indicate alternative pathways for direct HCys formation.

the enzymes are not studied, regulatory aspects are difficult to judge. So far one has to assume that due to the relation to bacteria kinetics and regulation are similar (Fig. 1).

It has to be mentioned that several organisms such as yeast or cyanobacteria evolved shunt pathways in which the reduced S-moiety of Cys is directly incorporated to form homocysteine (HCys) via HCys synthase (HS) (Fig. 2). HS is a more general term encompassing two individual enzyme activities, O-acetyl homoserine sulfhydrylase and O-succinyl homoserine sulfhydrylase, starting from succinyl or acetyl homoserine as precursors, respectively (Gophna et al., 2005). However, for plants such an ortholog has not been identified. It is reported that CGS possesses such a side activity (Hesse et al., 2004).

II. Biosynthesis of Methionine Precursors via Alternate Pathways

Intermediates in the synthesis of Met from its precursor homoserine, also a precursor to Thr synthesis, are shown in Fig. 2. There are alternate reactions at each step of the pathway, but the intermediates appear to be conserved for Met synthesis. The first step, homoserine activation, can occur in three ways: the first is acetylation, which is catalyzed by homoserine trans-acetylase (HTA) using acetyl-CoA as a substrate. This reaction is carried out by many bacteria such as *Leptospira meyeri* (Bourhy et al., 1997) or *Corynebacterium glutamicum* (Park et al., 1998) and fungi such as *Saccharomyces cerevisiae* (Yamagata, 1987) or *Neurospora crassa* (Kerr and Flavin, 1970) or cyanobacteria. Orthologs of the *met*2 gene (in analogy to the yeast gene) can be identified for the cyanobacterial strains *Chlorobium tepidum* TLS, strains of *Prochlorococcus marinus* MED4, SS120, and MIT9313, *Synechococcus* sp. WH8102, and *Rhodospeudomonas palustris* CGA009. The second is succinylation, which is catalyzed by homoserine trans-succinylase (HTS) utilizing succinyl-CoA as a substrate. This activity is known in some bacteria such as *Escherichia coli* (Rowbury and Woods, 1964) or *Pseudomonas aeruginosa* (Foglino et al., 1995). Here again orthologs for this gene can be identified in the genomes of *Prochlorococcus marinus* MED4, SS120, and MIT9313, and *Rhodospeudomonas palustris* CGA009. The third alternative reaction is phosphorylation, catalyzed by homoserine kinase (HSK). HSK catalyzes the formation of OPHS from homoserine and is converted in plants in a competing reaction by either CgS or Thr synthase (TS) either by condensation of Cys and OPHS to cystathionine being further converted to Met or directly to Thr (Thr) by TS. Bacteria use OPHS exclusively as precursor for Thr synthesis (Kredich, 1996). In plants HSK is much better characterized than in cyanobacteria. However, due to its low abundance it was not investigated in great detail in the past. The native

enzyme from wheat was purified to homogeneity and characterized (Riesmeier et al., 1993). The regulation of the enzyme is variable. Wheat HSK is not inhibited by physiological concentrations of Thr, Met, valine, isoleucine, or SAM (Riesmeier et al., 1993) while older studies revealed that HSK, both from pea and radish, is allosterically inhibited by these amino acids (Thoen et al., 1978; Baum et al., 1983). Greenberg et al. (1988) isolated soybean cell lines with repressed HSK activity resulting in Met accumulation. HSK is localized in plastids of pea probably containing soluble and membrane-associated HSK activities (Muhitch and Wilson, 1983; Wallsgrove et al., 1983). The localization was supported by the isolation of HSK in Arabidopsis. A single gene locus was identified and the predicted enzyme was localized in plastids with a mature mass of 33 kD. The recombinant protein was characterized biochemically to be dependent on Mg^{2+} and K^{+} ions (Lee and Leustek, 1999). The respective orthologs can be identified for *Synechocystis* sp. PCC6803, *Anabaena* sp. PCC7120, *Thermosynechococcus elongates* BP-1, *Gloeobacter violaceus* PCC7421, *Chlorobium tepidum* TLS, strains of *Prochlorococcus marinus* MED4, SS120, and MIT9313, *Synechococcus* sp. WH8102, and *Rhodospeudomonas palustris* CGA009.

Although the cognate activities HTA, HSK and HTS in general correspond to one of each of these protein families, evidence from in vitro experiments suggests that membership in a protein family does not unerringly predict substrate specificity. For example, *P. aeruginosa*, an HTS as defined by its activity in vitro is evolutionarily related to the HTA protein found in *Haemophilus influenzae* and not to the *E. coli* enzyme HTS (Foglino et al., 1995). Similarly, *Bacillus subtilis* which is known to possess homoserine transacetylase activity (Brush and Paulus, 1971) is homologous to the *E. coli* HTS (Rodionov et al., 2004). Thus, it is not a priori predictable to which group the respective orthologs from cyanobacteria belong because the sequence homology between both types of transacetylases is relatively high. For example, the annotation predictions of enzymes for *Rhodospeudomonas palustris* CGA009 suggest both, an HTS and an HTA function. This has to be proofed in future by biochemical investigations.

Incorporation of sulfur, the second step of the Met pathway, may also proceed in one of three ways (Fig. 2). Direct incorporation of sulfide (sulfhydrylation) into O-acetyl homoserine by O-acetyl homoserine sulfhydrylase (OAHS) to form HCys is found in several bacteria, fungi, and cyanobacteria (Kerr, 1971; Hwang et al., 2002; CyanoBase). HCys can also be synthesized by a second sulfhydrylation alternative, involving the direct incorporation of sulfide into a different substrate, O-succinyl homoserine, by O-succinyl homoserine sulfhydrylase (OSHS). This activity is found in some bacteria such as *Pseudomonas* species and cyanobacteria (Foglino et al., 1995; Vermeij and Kertesz, 1999; CyanoBase). Contradictory is the fact that cyanobacteria expressing HTS contain also a gene encoding OAHS indicating a still not complete and correct annotation of the genome.

III. Transsulfuration Process to Form Homocysteine

The transfer of the sulfur atom from Cys to HCys through the transsulfuration pathway with cystathionine as intermediate occurs only in plant plastids and has been extensively studied in Arabidopsis (Hesse et al., 2004 and references therein). The first committed step of de novo Met synthesis is a transsulfuration process via a γ-replacement reaction using Cys as the sulfur donor and O-phosphohomoserine as carbon precursor (Figs. 1 and 2). The formation of the thioether cystathionine is catalyzed by cystathionine γ-synthase (CGS) transferring reduced sulfur to a carbon backbone followed by a β-elimination carried out by cystathionine β-lyase (CBL) yielding HCys (Hesse and Höfgen, 2003; Hesse et al., 2004). The carbon/nitrogen precursor of Met synthesis in plants is distinct from that in yeast and bacteria. In yeast, Met is synthesized by direct sulfydration of O-acetylhomoserine; in bacteria, it is through a different pathway with succinyl-homoserine as substrate. In addition, the mature plant CGS exhibits at its N-terminus an additional module of about 120 amino-acids. This particular extension plays a role in its regulation (see section Regulation; Onouchi et al., 2004). Biochemical and kinetic parameters for CGS from diverse sources were extensively analyzed (Hesse et al., 2004 and references therein). The structure, substrate specificity and ping-pong

mechanism was recently confirmed through the resolution and analysis of the *Nicotiana tabaccum* CGS (Steegborn et al., 1999). Physiochemical properties of the second enzyme of the transsulfuration pathway (CBL) were relatively similar to those of *Escherichia coli* (Ravanel et al., 1996, 1998). Interestingly, while the bacterial enzyme degrades cystine and cystathionine equally, the reaction with cystine accounted for only 16% with the plant enzyme (Ravanel et al., 1996). Such observations suggest major differences in the amino acids close to the pyridoxal phosphate binding site between the bacterial and plant CBL, as confirmed through resolved structures from both sources (Clausen et al., 1996; Breitinger et al., 2001). The degree of homology CGS is also evident for cyanobacteria as in the genomes of the *Prochlorococcus marinus* strains MED4, SS120, and MIT9313, and the *Synechococcus* strain sp. WH8102 genes encoding CGS can be identified. However, due to rather high homology between CGS and CBL it is not possible to differentiate between both enzymes without having proofed their biochemical properties. Moreover, for *Anabaena* sp. PCC7120, *Chlorobium tepidum* TLS, and *Rhodospeudomonas palustris* CGA009 genes encoding the respective yeast enzymes such as cystathionine β-synthase and cystathionine γ-lyase are present. Here again it is a matter of annotation of these particular genes. All the enzymes for sulfur assimilation described above as well as the reverse transsulfuration enzyme cystathionine γ-lyase (CGL) belong to the same evolutionary family (henceforth the CGS family) and sequence alone may not reliably predict enzymatic activity. It can be assumed that some organisms having an active copy of each CGS, CBL and HS (Hwang et al., 2002; Ruckert et al., 2003), in cases where an organism has both direct incorporation and transsulfuration capacities, the same enzyme can carry out both activities (CGS as well as HS) (Vermeij and Kertesz, 1999; Auger et al., 2002; Hacham et al., 2003). Furthermore, substrate flexibility has also been demonstrated for some of these enzymes—so that a plant enzyme for example, which naturally metabolizes homoserine- phosphate, can also use acetyl-homoserine and succinyl-homoserine to form HCys directly (Thompson et al., 1982a; Kreft et al., 1994; Ravanel et al., 1995, 1998; Hacham et al., 2003). However, this alternative pathway seems to have only a minor physiological significance in plant cell metabolism regarding the entry of reduced sulfur into Met biosynthesis because HCys formation only takes place in the absence of Cys, and secondly, this direct sulfydration pathway participates in only 3% of total HCys synthesis and has seemingly no physiological significance (Giovanelli et al., 1978; MacNicol et al., 1981; Thompson et al., 1982a, b).

IV. Formation of Methionine by Transmethylation

The last step of Met synthesis is catalyzed by Met synthase (MS), which methylates HCys to form Met using N5-methyltetrahydrofolate as a methylgroup donor (Fig. 2). The function of this enzyme is on the one hand the de novo synthesis of Met and on the other the regeneration of the methyl group of S-adenosylmethionine. Thus, this step is essential even in organisms that do not synthesize Met, for the regeneration of the methyl group of SAM (Ravanel et al., 1998; Eckermann et al., 2000; Droux, 2004; Hesse et al., 2004). Using 5-methyltetrahydropteroyl glutamates as substrates, methylation of HCys can occur using two non-homologous enzymes: the cobalamin-dependent Met synthase (CDMS) in mammals, protists and most bacteria (Krungkrai et al., 1989; Goulding and Matthews, 1997; Evans et al., 2004) or the cobalamin-independent Met synthase (CIMS) as found in Bacteria including cyanobacteria (Whitfield et al., 1970; Cyanobase), *Viridiplantae* (Eichel et al., 1995; Zeh et al., 2002) and fungi (Kacprzak et al., 2003). Orthologs of CDMS have not been identified so far in cyanobacteria. However, it can be assumed that *met*E orthologs are present in cyanobacterial genomes. Since it is known from a survey of 326 algal species that approximately half of them require exogenous vitamin B_{12} because they are cobalamin auxotrophs it has to be assumed that the source of cobalamin seems to be bacteria, indicating an important and unsuspected symbiosis (Croft et al., 2005). Thus, it can be assumed that cyanobacteria have the capability to synthesize cobalamin as bacteria have. So far, the molecular and biochemical characterization of Met synthase from plants is still limited.

One reason for this is the low amount of protein present in plants and another is the substrate specificity of the enzyme. While bacteria are able to use monoglutameric methyltetrahydrofolate, *Catharanthus roseus* (Eichel et al., 1995) accepts only the triglutameric isoform that is not freely available. Neither SAM nor cobalamin is required for activity of the cobalamin-independent Met synthase as demonstrated for MS from *C. roseus* and *S. tuberosum* (Eckermann et al., 2000; Zeh et al., 2002). Intensive studies on MS expression revealed that MS is a low copy gene differentially expressed, at least in potato organs, with elevated levels in flowers, basal levels in sink and source leaves, roots, and stolons, and low levels in stems and tubers. This is in good agreement with protein data except that the protein content in leaves was less than expected from the RNA data. Western blot analysis of subcellular fractions revealed that the protein is located in the cytosol. However, the changing pattern of gene expression during the day/night period implied a light-dependent control of MS-transcription normally seen for enzymes localized in plastids. The expression of MS was shown to be light-inducible with its highest expression at midday, while, during the night, expression dropped to a low, basal mRNA level. These RNA data were not confirmed at the protein level since the protein content remained constant over the whole day. Feeding experiments using detached leaves revealed that sucrose or sucrose-derived products are responsible for the induction of StMS1 gene expression, but with no effect on the protein level in *C. roseus* and *S. tuberosum* (Eckermann et al., 2000; Nikiforova et al., 2002; Zeh et al., 2002). Multiple forms of MS could be identified in plants, and in the genome of *A. thaliana* (Eckermann et al., 2000; Droux, 2004; Hesse et al., 2004; Ravanel et al., 2004). Physicobiochemical investigations were performed to answer the localization of the final synthesizing step. Three *Arabidopsis thaliana* Met synthase (MS) were identified from which two are cytosolically and one plastidial localized (Ravanel et al., 2004). It could be further shown that the plastid isoform exhibited a stronger affinity for the polyglutamate derivatives of folates than the cytosolic isoforms being probably the reason not having detected the plastidial activity earlier (Ravanel et al., 2004). The plastid localization of MS corroborated earlier observations based on enzyme measurements having been made in pea chloroplasts and mitochondria suggesting that the plastidial de novo synthesis of Met is favored indicating the autonomy of the compartment (Shah and Cossins, 1970; Clandinin and Cossins, 1974). By contrast, the photosynthetic protozoan *E. gracilis* Z. expresses three isoforms of cobalamin-dependent enzymes located in chloroplasts, mitochondria, and cytosol in addition to a cytosolic cobalamin-independent Met synthase (Isegawa et al., 1994).

V. Methionine Recycling

Met is a sulfur-containing amino acid that can be activated by ATP to S-adenosyl-Met (SAM). Radiotracer experiments indicate that more than 80% of the label from ^{14}C-methyllabeled Met was incorporated into lipids, pectines, chlorophyll, and nucleic acids, whereas less than 20% in protein (Giovanelli et al., 1985). Thus, apparently the majority of Met is converted into SAM for transmethylation reactions in plants (Fig. 3). SAM is synthesized from Met and ATP by the enzyme SAM synthetase from which three isoforms exists in plants (Shen et al., 2002). SAM serves as a substrate in many biochemical reactions. The high need for SAM makes it necessary to recycle Met. When SAM is utilized for the synthesis of ethylene, certain polyamines, and siderophores, methylthioadenosine (MTA) is produced as an intermediate which can be recycled to Met and allows high rates of the before mentioned metabolites (Bürstenbinder et al., 2007). This Met salvage pathway was first characterized and was described in plants at the biochemical level and is termed according to the discoverer Yang or MTR cycle (Wang et al., 1982; Yang and Hoffman, 1984; Miyazaki and Yang, 1987). However, this circuit is not unique for plants and was identified also in bacteria, including cyanobacteria, and animals. Although the major progress in detailing enzyme characteristics and in identifying their corresponding genes has come from work on prokaryotes (Sufrin et al., 1995; Cornell et al., 1996; Sekowska et al., 2001; Sekowska and Danchin, 2002), the plant field gain on more and more information (e.g., Sauter et al., 2004, 2005; Bürstenbinder et al., 2007). In bacteria and in plants, MTA is depurinated to 5-methylthioribose (MTR) through

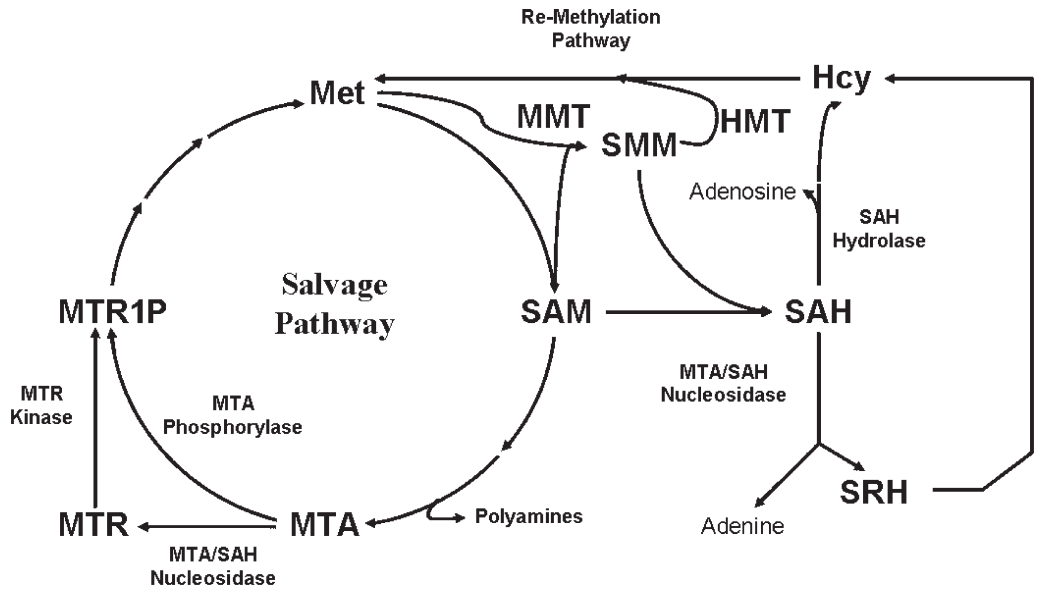

Fig. 3. Met salvage pathway in plants and microorganisms. Met, methionine; MMT, Met S-methyltransferase; HMT, HCys S-methyltransferase; SRH, S-ribosylhomocysteine; MTA, methylthioadenosine; MTR, 5′-methylthioribose; MTR1P, 5′-methylthioribose 1-phosphate; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; SMM, S-methylmethionine.

the enzymatic activity of MTA nucleosidase. MTR kinase catalyzes the subsequent phosphorylation of the C-1 hydroxyl group of the Rib moiety of MTR to yield 5-methylthio- Rib-1-P. In animals, MTA phosphorylase carries out both functions in a single ATP-independent step (Schlenk, 1983). Hence, MTR kinase exists in prokaryotes and plant species but not in animals. 5-Methylthio- Rib-1-P subsequently undergoes enzymatic isomerization, dehydration, and oxidative decarboxylation to 2-keto-4-methylthiobutyrate, the immediate precursor of Met (Miyazaki and Yang, 1987). The second possibility to recover Met is the recycling of HCys resulting from hydrolysis of S-adenosylhomocysteine (AdoHCys), produced from cytosolic SAM-dependent methylation (Kloor and Osswald, 2004). It should be noted that AdoHCys is a strong inhibitor of methylation steps and, thus, needs to be quickly transformed into HCys and eventually Met again. Furthermore, accumulation of the hydrolytic product (HCys) of the reaction catalyzed by SAH hydrolase is reversible (Kloor et al., 1998; Kloor and Osswald, 2004). Thus, HCys will be eliminated through degradation, transport to other compartments, or through direct conversion into Met, the later step being catalyzed by the cytosolic Met synthase (Ravanel et al., 1998, 2004). Furthermore, Met is converted to S-methylmethionine (SMM) which appears to be a phloem localized transport form of reduced S in plants, as is GSH (Ranocha et al., 2001; Kocsis et al., 2003). The SMM to GSH ratio varies between different plants. SMM is synthesized from Met and SAM by SAM:Met S-methyltransferase releasing S-adenosylhomocysteine (MMT). SMM can be reconverted to Met by HCys S-methyltransferase (HMT) methylating HCys yielding two molecules of Met. Essentially this appears to be a shortcut of the SAM methylation cycle and currently it is speculated that its main function is the down regulation of SAM levels in plants. However, as SMM is also transported in the phloem it might well contribute to reduced sulfur supply to sinks. Furthermore, this mechanism presents a second system to remove the excess of HCys produced in the cytosol when folate derivatives are insufficient for Met synthesis. SMM is present in millimolar concentration in plants and constituted storage for sulfur and methyl groups, as well a system to maintain the cell SAM level constant as was demonstrated in mutant plants with inactivated Met S-methyltransferase (Kocsis et al., 2003). The above mentioned process has not been investigated in detail for cyanobacteria. Analysis of the genome of cyanobacteria revealed that most of the genes are present leading to the assumption that the processes as described

above for microorganisms are also functional in cyanobacteria. The major difficulty as already discussed above is the weakness of annotation of open reading frames. SAM synthetase is present in nearly all cyanobacteria indicating, that the initial step, the formation of SAM, is performed. However, somewhat problematic is the classification of genes encoding enzymes of the Yang cycle or of the HCys recycling pathway. Only few genes of the cycle are annotated, however, providing evidence that both recycling pathways are existent. It is also not evident whether cyanobacteria, although living partially in sea water, are able to form dimethylsulfiopropionic acid (DMSP) which derives from SMM as the respective genes were not identified so far. DMSP is an excellent compatible solute widely used for osmoprotection in bacteria and algae and for cryoprotection in algae (Kiene et al., 1996; Malin and Kirst, 1997; Stefels, 2000; Welsh, 2000; see also Chapters 14 and 22, this issue).

VI. Regulatory Aspects of Methionine Homeostasis

The synthesis of Met is subject to a complex regulation (Fig. 4). The whole pathway is regulated by feedback inhibition of aspartate kinase by either lysine or Thr or additionally lysine synergistically together with S-adenosylmethionine (SAM). Earlier studies have shown that the provision of the carbon backbone, e.g., by over-expression of aspartate kinase increased the synthesis of lysine and Thr leading to the assumption that amino acid pools are filled in a controlled order (Galili, 1995; Hesse and Hoefgen, 2003; Hesse et al., 2004; Galili et al., 2005). This result suggests that the carbon flux is limiting the synthesis of Met. Insights into the regulation of the Met network were achieved with a variety of transgenic approaches targeted at the various steps of the transsulfuration pathway, including the cytosolic Met synthase,

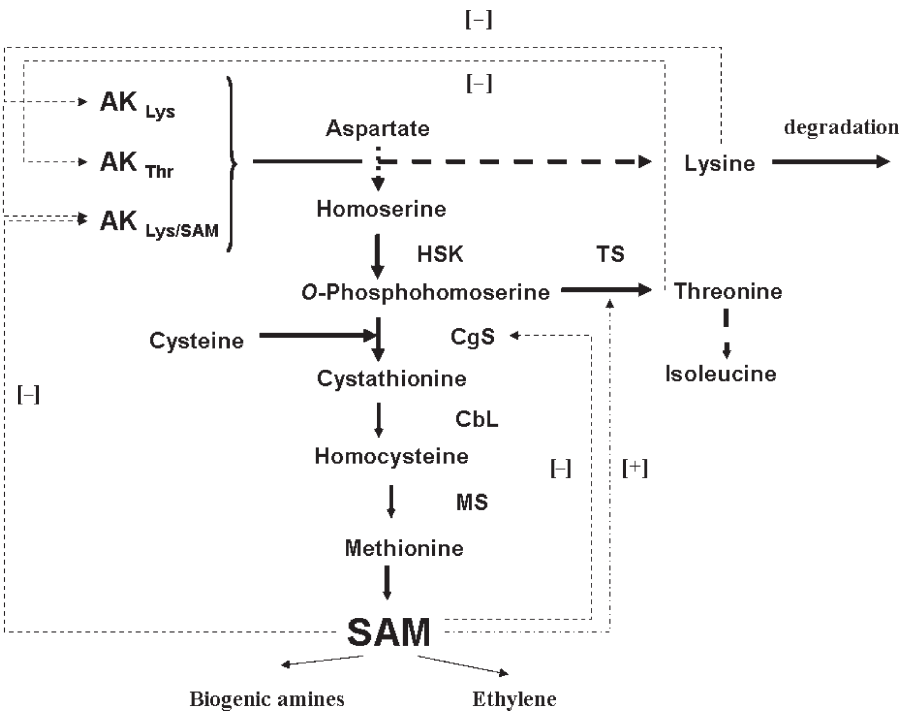

Fig. 4. Schematic diagram of the metabolic networks regulating Lysine, Thr and Met metabolism. Only some of the enzymes and metabolites are specified. Dashed arrows with a 'minus' symbol represent either feedback inhibition loops or repression of gene expression. The dashed and dotted arrow with the 'plus' symbol represents the stimulation of gene expression or enzyme activity. Abbreviations: AK, aspartate kinase (sensitivity to metabolites are indicated); HSK, homoserine kinase; TS, threonine synthase; CGS, cystathionine γ-synthase; CBL, cystathionine β-lyase; MS, Met synthase; SAM, *S*-adenosyl Met. Dashed lines indicate multiple enzymatic steps.

and also the enzymes synthesizing or competing for the O-phosphohomo- serine substrate, HSK and TS, respectively (Hesse and Hoefgen, 2003; Droux, 2004; Hesse et al., 2004; Lee et al., 2005; Rinder et al., 2007). Conclusion of theses studies revealed that in Arabidopsis CGS was the unique enzyme controlling the flux of sulfur and of the carbon–nitrogen backbone toward Met synthesis either at the level of CgS on RNA level (CgS in Arabidopsis; Chiba et al., 1999) or TS activity level by SAM (TS in Arabidopsis and potato; Curien et al., 1996, 1998; Zeh et al., 2001) depending on the plant species (Hesse and Hoefgen, 2003; Hesse et al., 2004). TS activity is positively regulated by SAM, a direct product of Met, thus favoring carbon flow into Thr biosynthesis in preference to Met synthesis when sufficient SAM is available. Under these conditions the K_M-values of TS for OPHS have been shown to be 250- to 500-fold lower as compared to the competing enzyme, CgS, thus favoring carbon flow into Thr biosynthesis in preference to Met synthesis (Madison and Thompson, 1976; Curien et al., 1996, 1998; Laber et al., 1999). A long standing question was answered recently how SAM is able to activate TS. Now, there is evidence for a counter-exchange transporter of AdoHCys from the plastid to the cytosol with cytosolic synthesized SAM (Ravanel et al., 2004) and SAM transporter (Bouvier et al., 2006) However, it is questionable whether a complete shut down of Met synthesis is reached. The demand of SAM for several processes has to be assumed to be high indicating that most of the synthesized SAM is used. Only a small part is available to be translocated into chloroplasts in order to activate TS. Thus, it can be assumed that carbon provided by aspartate via OPHS is preferentially used to form Met subsequently used for SAM synthesis because in a non-activated condition the K_M value for the substrate is lower compared to TS. Feeding studies with $^{13}CO_2$ revealed that the carbon flux is priorly directed to Met (and, hence, SAM) formation within this pathway and even when compared to other pathways, while lysine and Thr/isoleucine pools provide minor sinks (Hesse, Schauer, Hoefgen, Fernie, unpublished; Fig. 5). From this point of view CGS is the most important enzyme for Met synthesis. As demonstrated by feeding studies, CgS is not an allosteric enzyme since enzyme activity is not inhibited by Met (Thompson et al., 1982b). Mutants and transgenic plants have been used to display the central role of CgS in the synthesis of Met in Arabidopsis. Most extensive developments on the molecular regulation of the Met network were investigated by characterization of *Arabidopsis thaliana* mutant lines resistant to the toxic analogue of Met, ethionine. These mutant lines called *mto*1(1–7) were characterized by elevated levels of CGS mRNA and of CGS activity as compared to wild type (Onouchi et al., 2004) (Fig. 6). Genetic analysis described a mutation within the conserved exon1 coding region (*mto*1 region) where a single nucleotide changes resulted in one amino acid modification (Chiba et al., 1999, 2003; Amir et al., 2002). This important motif acts in cis to stabilize the CGS mRNA being resistant to degradation (Suzuki et al., 2001; Ominato et al., 2002; Onouchi et al., 2004). Translation of the protein is necessary for the regulation and stability of the mRNA as was confirmed by an experiment using the in vitro translation system of wheat germ extract (Chiba et al., 2003; Lambein et al., 2003). This result is supported by the recent finding for tomato fruits during ripening showing that Met down-regulates CGS transcript levels (Katz et al., 2006). However, this finding is inconsistent to the earlier finding for potato CgS transcript stability which is not influenced by Met (Kreft et al., 2003). Finally, since SAM was revealed as the crucial metabolite involved in this regulation, suggesting that the Met dependent regulation was the result of its transformation into SAM in the cytosol (Onouchi et al., 2004). Interestingly, the mto1 domain showed no features of known SAM binding domains. From this observation, it may be speculated that SAM regulation takes place through a protein intermediate. From the recent investigation using wheat germ extract, one could conclude that this intermediate is constitutively expressed (Chiba et al., 2003; Kreft et al., 2003; Onouchi et al., 2004). A mechanism how SAM might regulate CGS stability is suggested by Onouchi et al. (2005). They suggest a temporary arrest in the translation process at serine-94 (nucleotide 280 from the first ATG of the mRNA) when the SAM level increased which strongly correlates between this arrest and mRNA degradation (Onouchi et al., 2005). Very recently it could be demonstrated that in addition to the full-length CGS transcript, a deleted form exists in Arabidopsis (Hachem et al., 2006). The

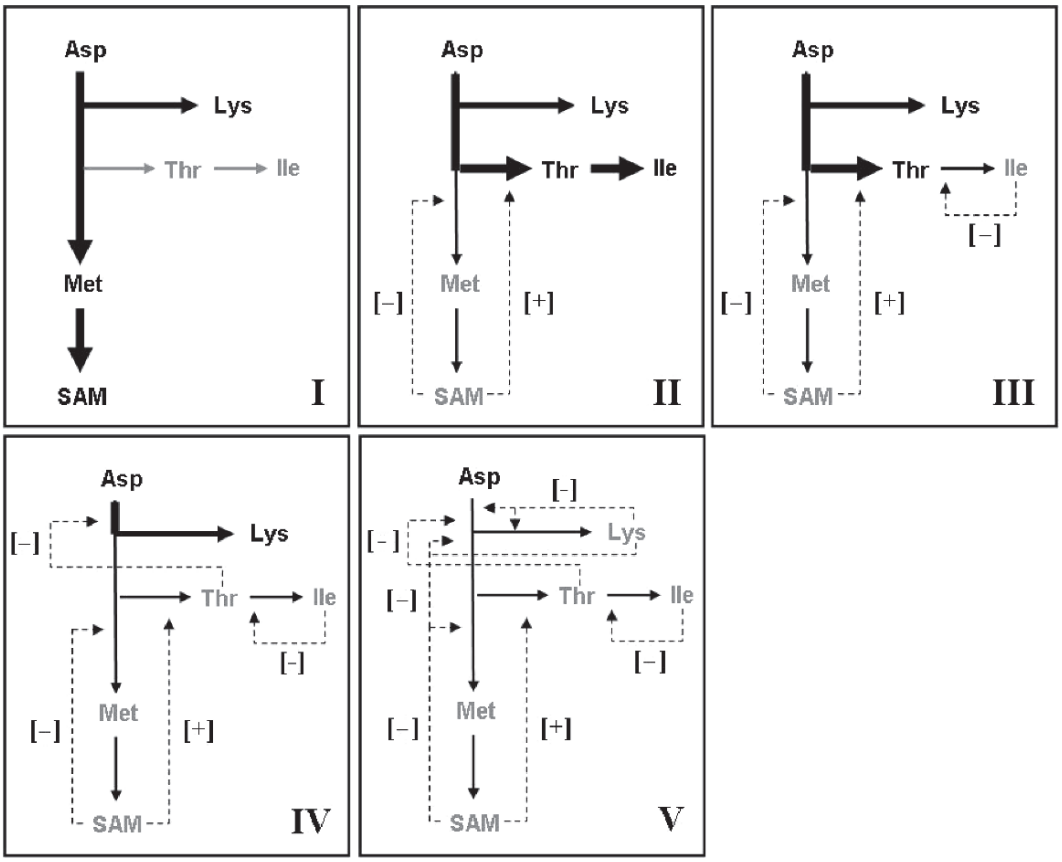

Fig. 5. Schematic presentation of carbon flow and feedback control mechanism in Arabidopsis as model plant. Boxes I–V represents the dynamic regulation of the pathway via product inhibition. Thickness of the arrows indicates the carbon flow in the pathway. Dashed arrows with a 'minus' symbol represent either feedback inhibition loops or repression of gene expression. The dashed arrow with the 'plus' symbol represents the stimulation of gene expression or enzyme activity.

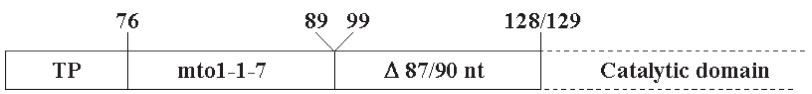

Fig. 6. The Arabidopsis mRNA of CGS is shown schematically. TP, the chloroplast-targeting transit peptide; mto1 domain indicates the region with point mutations; Δ, indicates the deleted region of 87 or 90 bp; catalytic domain, the catalytic part of CGS that is highly homologous to bacteria CGSs. Nucleotides are numbered from the ATG initiator codon.

deleted transcript of CGS that lacks 90 or 87 nt located internally in the regulatory N-terminal region of CGS localized adjacent to the *mto*1 region maintains the reading frame of the protein (Fig. 6). The deleted region is located within the first exon of the CGS transcript and is not flanked by any known consensus intron/exon boundary sequences, suggesting that the shorter transcript is not a result of consensus alternative splicing. At the transcript level, it is highly enriched for G-C content (over 70%), suggesting a possible secondary structure (Fig. 7A). When overexpressed in transgenic tobacco plants the transgenic plants accumulated Met to a much higher level than those that expressed the full-length CGS probably because this form of CGS is not subject to feedback regulation by Met, as reported for the full-length transcript (Hachem et al., 2006).

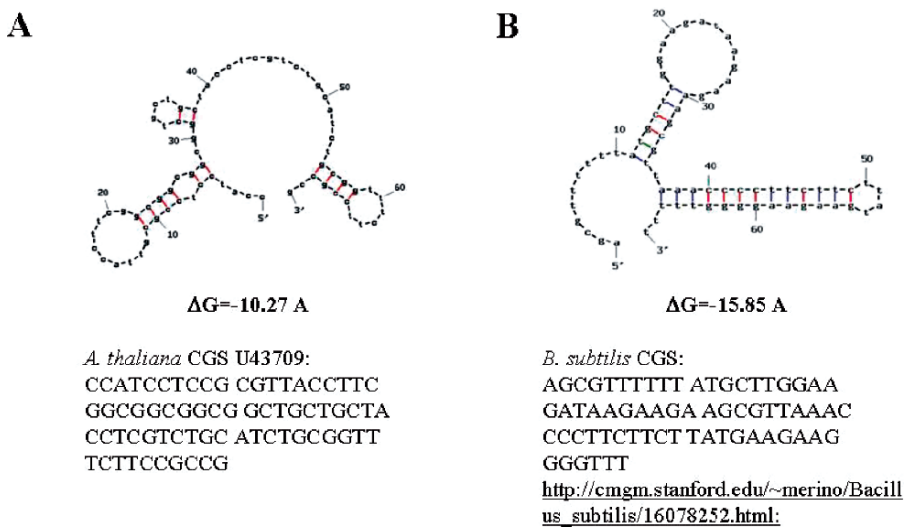

Fig. 7. Prediction of secondary RNA structure for the 90-nt deleted region from Arabidopsis (A) in comparison to *B. subtilis* (B). The predicted calculated ΔG of this stem-loop structure is −10.27 Kcal mol^{-1} for Arabidopsis and −15.85 Kcal mol^{-1} for *B. subtilis*. The prediction was calculated using the software located on the following webpage: www.bioinfo.rpi.edu/applications/hybrid/quikfold.php.

The regulation of Met biosynthesis in cyanobacteria has not been investigated in the past. However, since the genomes of different cyanobacteria ecotypes are known and for most of the enzymatic steps respective genes were identified, one can assume that the controlling mechanisms function nearly identical. Met biosynthesis in *E. coli* has been investigated in details (e.g., for review: Kredich, 1996). Two types of control can be identified in bacteria, the control of gene expression and secondly, the metabolite control mechanism. For *E. coli* transcription nearly all the *Met* genes involved in Met biosynthesis, except *metH*, are under negative control of the MetJ repressor. MetJ interacts with *S*-adenosylmethionine, the pathway's end product, as a corepressor (Saint-Girons et al., 1988). Expression of the *metE, metA, metF, metH*, and *glyA* genes is additionally under positive control of the MetR activator (Maxon et al., 1989; Urbanowski and Stauffer, 1989; Weissbach and Brot, 1991; Mares et al., 1992; Cowan et al., 1993; Lorenz and Stauffer, 1996). HCys may modulate the regulator role of MetR and is required for the *metE* gene activation (Urbanowski and Stauffer, 1987). Furthermore, vitamin B_{12} is involved in *metE* repression, probably by depletion of the coactivator HCys (Wu et al., 1992). MetR is a member of the LysR family of prokaryotic transcriptional regulatory proteins. Common family features are the size (between 300 and 350 amino acids), the formation of either homodimers or homotetramers, the presence of a helix-turn-helix DNA binding motif in the N-terminal region, and the requirement for a small molecule that acts as a co-inducer (Schell, 1993). MetR activates gene expression at one or more loci while negatively regulating the expression of their own genes. In other microorganisms such as *Bacillus subtilis, Clostridium acetobutylicum*, and *Staphylococcus aureus*, several genes involved in the biosynthesis of Met are regulated by a global transcription termination control system called the S box regulon (Grundy and Henkin, 1998). The S box belongs to a group of regulatory elements, so called riboswitches. Riboswitches are RNA elements in the 5′ untranslated leaders of bacterial mRNAs that directly sense the levels of specific metabolites with a structurally conserved aptamer domain to regulate expression of downstream genes. Riboswitches use the untranslated leader in an mRNA to form a binding pocket for a metabolite that regulates expression of that gene. Riboswitches are dual function molecules that undergo conformational changes and that communicate metabolite binding typically as either increased transcription termination or reduced translation efficiency via

an expression platform (Breaker, 2002; Winkler and Breaker, 2005). So far identified in *B. subtilis* are target genes involved in vitamin B1, B2, and B12 synthesis and transport via *rfn*-, *thi*-, and B_{12}-box, respectively, SAM via the *S*-box, lysine via the *L*-box, and guanine/hypoxanthine via the *G*-box (Nudler and Muronov, 2004). Although not investigated in detail for gram-negative bacteria *S*-boxes play a major role in the regulation of Cys and Met synthesis in gram-positive bacteria such as *Bacillus subtilis*. The S-box regulatory system is used in gram-positive organisms, including members of the *Bacillus/Clostridium/Staphylococcus* group, to regulate expression of genes involved in biosynthesis and transport of Met and *S*-adenosylmethionine (SAM) (Grundy and Henkin, 2004; Rodionov et al., 2004) (Fig. 8). Genes in the S-box family exhibit a pattern of conserved sequence and structural elements in the 5′ region of the mRNA, upstream of the start of the regulated coding sequence(s). These conserved elements include an intrinsic terminator and a competing antiterminator that can sequester sequences that otherwise form the 5′ portion of the terminator helix; residues in the 5′ region of the antiterminator can also pair with sequences located further upstream, and this pairing results in formation of a structure (the anti-antiterminator) that sequesters sequences necessary for formation of the antiterminator. Genetic analyses of S-box leader RNAs supported the model that formation of the anti-antiterminator and transcription termination occur during growth under conditions where Met is abundant, while starvation for Met results in destabilization of the anti-antiterminator, allowing antiterminator formation and read-through of the transcription termination site (Grundy and Henkin, 1998). Overexpression of SAM synthetase in vivo results in increased repression of S-box gene expression, supporting the model that SAM is the effector in vivo (Auger et al., 2002; McDaniel et al., 2003). These results suggested that S-box RNAs directly sense SAM and are therefore members of the family of RNA elements termed 'riboswitches,' which monitor regulatory signals without a requirement for accessory factors such as RNA-binding proteins or ribosomes (Nudler and Mironov, 2004; Tucker and Breaker, 2005). The binding of SAM at the regulatory RNA element could be demonstrated by co-crystallization of an S-adenosylmethionine-responsive riboswitch from *Thermoanaerobacter tengcongensis* with SAM (Montagne and Batey, 2006). Also very recently five novel structured RNA elements were identified by focusing comparative sequence analysis of intergenic regions on genomes of α-proteobacteria (Corbino et al., 2005). One of the five newly found motifs from *Agrobacterium tumefaciens*, termed *metA*, appeared to function as a riboswitch that senses *S*-adenosylmethionine (SAM) (Fig. 8). This SAM-II riboswitch class has a consensus sequence and conserved structure that is distinct from the SAM-I riboswitch reported previously (Epshtein

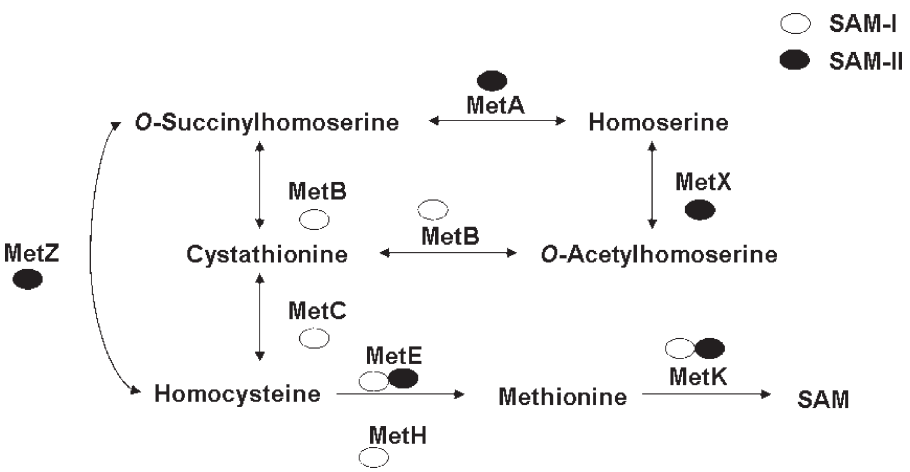

Fig. 8. Comparison of genes in the Met and SAM biosynthetic pathways found downstream of SAM-I and SAM-II riboswitches in gram-negative and positive bacteria.

et al., 2003). Compared with SAM-I aptamers, SAM-II aptamers are smaller and form a simpler secondary structure. However, the SAM-II aptamer exhibits a level of molecular discrimination that is similar to that observed for the SAM-I riboswitch. These findings demonstrate that biological systems use multiple RNA motifs to sense the same chemical compound. Further detailed analyses have to be done in future to investigate this regulatory mechanism in *E. coli* and broaden it to other species such as members of the cyanobacterial family. As two SAM riboswitches systems are identified in bacteria one might argue that this mechanism might be 'RNA World' relics that were selectively retained in certain bacterial lineages or new motifs that have emerged since the divergence of the major bacterial groups. A third regulatory riboswitch was identified very recently in lactic acid bacteria (Fuchs et al., 2006). Here SAM synthetase (MetK) expression is regulated by RNA conformational changes in the 5′UTR due to SAM binding subsequently negatively regulating SAM synthesis. This brings us back to the regulatory mechanism in plants that SAM is able to modulate transcript stability. Perhaps, although CGS does not contain a so far known SAM binding domain, this regulatory mechanism might be a conserved relict. Taking into account that according to the endosymbiontic theory the plastidial targeting developed later than the regulatory mechanism via SAM one might speculate that the mto1 domain and the adjacent 90 nt might have evolved combining both, an open reading frame and a control mechanism via SAM (Fig. 7). Similarities between RNA stem loop structure of CGS from *A. thaliana* and *B. subtilis* are not to neglect. The evolutionary consequences of this finding have to be explored in the future.

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