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REVIEW ARTICLE

Current understanding of the factors regulating methionine content in vegetative tissues of higher plants

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Abstract Methionine is a nutritionally essential, sulfur-containing amino acid found in low levels in plants, which often limits its value as a source of dietary protein to humans and animals. Methionine is also a fundamental metabolite in plant cells since, through its first metabolite, *S*-adenosylmethionine (SAM), it controls the level of several key metabolites, such as ethylene, polyamines and biotin. SAM is also the primary methyl group donor that regulates different processes in plants. Despite its nutritional and regulatory significance, the factors regulating methionine content in plants are not fully known. In this review, we summarize the current knowledge and recent progress made in our understanding of the methionine metabolism. The enzymes and substrates that regulate methionine synthesis were described, as well as the influences of the catabolic pathways of methionine on its content. The current effort to tailor an improvement of methionine content in vegetative tissues with minimal interference in plant growth and productivity is described as well. The accumulated knowledge has provided new insights into the control of methionine level in plants and, in some cases, has resulted in significant improvements in the nutritional value of plants.

Keywords Aspartate family · Methionine metabolism · Methionine-rich storage proteins · Nutritional improvement · Regulation · *S*-adenosylmethionine

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The importance of methionine in plant metabolism

The sulfur-containing amino acid methionine is an essential amino acid, the level of which often limits the nutritional value of crop plants (Galili et al. 2005). Yet, aside from its nutritional importance, methionine is also a fundamental metabolite in plant cells. Apart from its role as a protein constituent and its central role in the initiation of mRNA translation, methionine indirectly regulates a variety of cellular processes such as the precursor of SAM, which is the primary biological methyl group donor (Fig. 1a). The substrate of SAM-dependent methyltransferases participates in both primary and secondary metabolism. Examples include lipids, DNA, RNA, proteins, pectin, alkaloids, phytosterols and osmoprotectants. SAM-dependent methyltransferases also participate in reactions required for chlorophyll synthesis, for lignins and suberins synthesis, in flavonoids, hydroxycinnamic acids and stilbenes, and in other aromatic as well as volatile fragrance and aroma compounds (Kagan and Clarke 1994; Roje 2006). Hence, as a donor for methyl groups, methionine through SAM regulates essential cellular processes such as cell division, cell wall synthesis, chlorophyll synthesis and membrane synthesis (Roje 2006). In higher plants, SAM is also the precursor for the hormone ethylene (Fig. 1b), which regulates developmental stages including ripening and senescence (Yang and Hoffman 1984; Yang et al. 1990; Matilla 2000). Moreover, SAM is the source of the propylamino group in the synthesis of the polyamines spermidine and spermine, which play crucial roles in many aspects of plant growth, including cell proliferation and differentiation, apoptosis, homeostasis and gene expression (Pang et al. 2007). SAM is also the precursor for the metal ion chelating compounds, nicotinamide and phytosiderophores, as well as the biotin co-factor (Ravanel et al. 1998; Droux 2004; Hesse et al. 2004; Roje 2006).

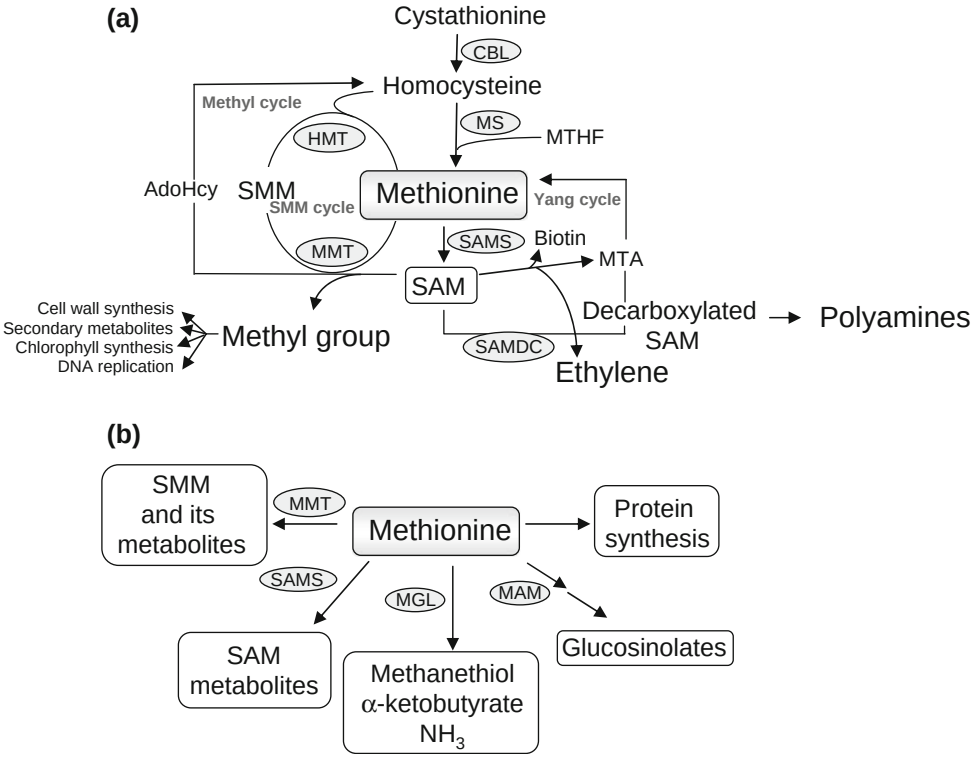

Fig. 1 **a** The three recycle pathways of methionine; **b** the methionine fate in higher plants. *CBL* cystathionine β-lyase; *MS* methionine synthase; *SAM* *S*-adenosylmethionine; *SAMS* SAM synthase; *SAMDC* SAM decarbosylase; *MTA* methylthioribose; *AdoHcy* adenosylhomocysteine; *SMM* *S*-methylmethionine; *MMT* methionine *S*-methyltransferase; *HMT* homocysteine *S*-methyltransferase; *MGL* methionine γ-lyase; *MAM* methyl thioalkylmalate synthases

In the Brassicaceae family, methionine itself serves as a donor for various secondary metabolites belonging to glucosinolates (Fig. 1b), which are involved in pathogen and insect defense (Gigolashvili et al. 2007; Hirai et al. 2007). Finally, methionine leads to the synthesis of *S*-methylmethionine (SMM) (Fig. 1b), which is considered to be the mobile and storage form of methionine (Mudd and Datko 1986), the regulator of SAM level in cells (Ranocha et al. 2001; Kocsis et al. 2003) and a precursor to other secondary metabolites such as 3-dimethyl-sulfonio-propionate, an osmoprotectant accumulated by certain flowering plants and algae (Hanson et al. 1994).

Due to the regulatory and nutritional importance of methionine, considerable efforts were invested to study the factors regulating its metabolism in plants, particularly in vegetative tissues (e.g., Ravanel et al. 1998; Kim et al. 2002; Kreft et al. 2003; Onouchi et al. 2004; Avraham et al. 2005; Golan et al. 2005), and much less in seeds. The highlights of the efforts made in vegetative tissues are described in the following review, which is divided into three sub-sections. First, it will describe the regulatory role of enzymes in the methionine biosynthesis pathway, as well as the roles of the carbon/amino skeleton, cysteine and the methyl group flow toward the biosynthesis pathway of methionine on the methionine level. Second, the regulatory role of the catabolic enzymes of methionine on its content will be described. Third, the importance of methionine as an essential amino acid for human nutrition will be described, as well as efforts to increase its level in the vegetative tissues of plants.

The regulatory role of enzymes and substrates in the methionine biosynthesis pathway on methionine level

The regulatory role of cystathionine γ-synthase

Cystathionine γ-synthase (CGS), the first unique enzyme of the methionine biosynthesis pathway, combines the carbon/amino skeleton derived from aspartate with the sulfur moiety derived from cysteine (Kim and Leustek 1996) (Fig. 2). Unlike other regulatory enzymes in the aspartate biosynthesis pathway whose activities are regulated by the feedback inhibition mechanism mediated by their products, CGS activity is not feedback inhibited by methionine or methionine metabolites (Ravanel et al. 1998). However, it was found that the transcript level of *Arabidopsis* CGS (AtCGS) was negatively regulated by the methionine downstream product, SAM, via a post-transcriptional mechanism (Chiba et al. 1999, 2003; Onouchi et al. 2004). This regulation occurs in the N-terminal region of AtCGS comprising $\sim$100 amino acids (without its plastid transit peptide), which does not exist in bacterial enzymes. This N-terminal region is not essential for the catalytic activity of AtCGS, but influences the levels of methionine and its

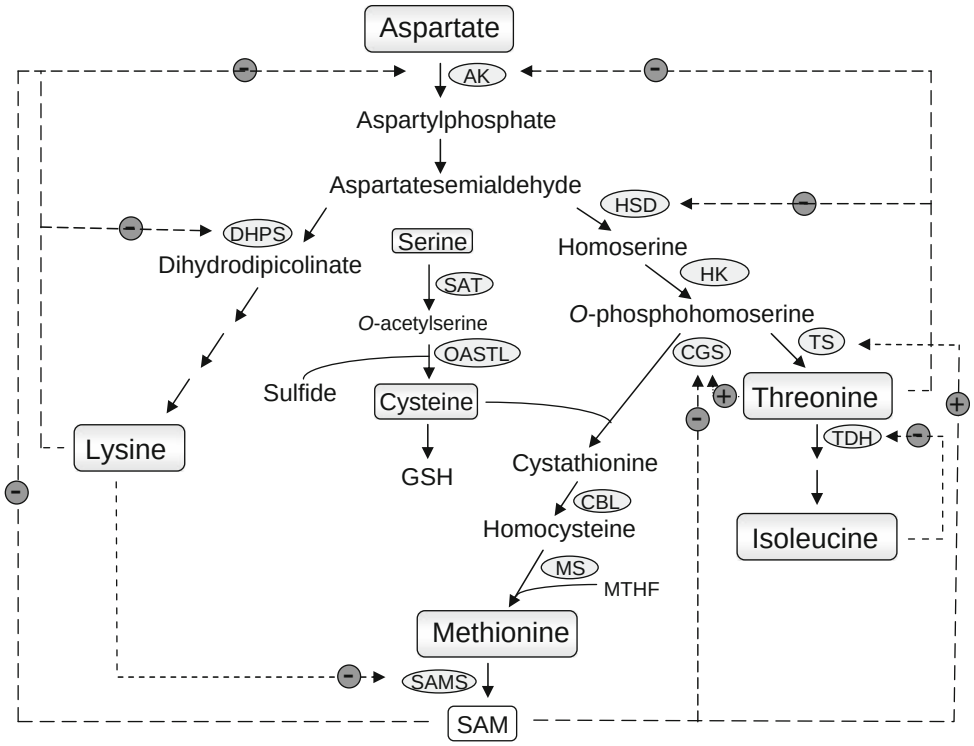

Fig. 2 The aspartate family of amino acids and the biosynthesis pathways leading to methionine and cysteine in higher plants. Only some of the enzymes and metabolites are specified. *Dashed arrows* with a "minus" sign represent either feedback inhibition loops or repression of gene expression. The *dashed* and *dotted arrow* with the "*plus*" sign represent the stimulation of gene expression or enzyme activity. *AK* Asp kinase; *DHPS* dihydrodipicolinate synthase; *HSD* homoserine dehydrogenase; *HK* homoserine kinase; *TS* threonine synthase; *TDH* threonine dehydratase; *OPH* *O*-phosphohomoserine; *CGS* cystathionine γ-synthase (marked in *red*); *CBL* cystathionine β-lyase; *MS* methionine synthase; *SAT* serine acetyltransferase; *OPH* *O*-phosphohomoserine; *OASTL* *O*-acetylserine (thiol)lyase; *GSH* glutathione; *SAM* *S*-adenosylmethionine; *SAMS* SAM synthase

metabolites in plants (Hacham et al. 2002). A set of mutations in a conserved sub-domain within the N-terminal region in *Arabidopsis* mutants (termed MTO1) led to a 40-fold higher methionine content compared to wild-type plants (Inba et al. 1994; Chiba et al. 1999). A detailed analysis of this MTO1 domain reveals that SAM induces a temporal arrest in the translation elongation process during AtCGS mRNA translation of this domain. As a result of the translation arrest, mRNA degradation occurs upstream of the stalled ribosome, resulting in the production of the 5′-truncated RNA species, and thus leading to decay of the transcript (Onouchi et al. 2005; Onouchi et al. 2008; Haraguchi et al. 2008).

Notwithstanding the regulatory post-transcriptional function of the MTO1 region, no inverse correlation between high methionine levels and low AtCGS mRNA levels was evident in transgenic *Arabidopsis* plants constitutively expressing the endogenous AtCGS (Kim et al. 2002). Moreover, overexpression of AtCGS in tobacco (Hacham et al. 2002, 2006), potato (Di et al. 2003) and alfalfa plants (Avraham and Amir 2005) showed a positive correlation between AtCGS level and methionine content. Whether these observations are due to variations in MTO1 machinery between different *Arabidopsis* tissues and plant species, or the result of the overexpression itself, must still be elucidated.

To gain more knowledge about the role of the conserved MTO1 domain in controlling the level of CGS in other plant species, tomato and potato plants were fed with high levels of methionine. These experiments showed that the CGS of tomato was sensitive to high methionine application (Katz et al. 2006) similar to that of *Arabidopsis,* while potato CGS1, which shares 97% identity with tomato CGS, was not affected by high methionine application (Hesse and Hofgen 2003). Moreover, overexpression of potato CGS in potato plants, even though it significantly increased enzyme activity, does not lead to a higher methionine level, implying that CGS in potato plants does not play a major role in controlling methionine level (Kreft et al. 2003). This observation suggests that potato lacks some elements required for post-transcriptional CGS regulation (Hesse and Hofgen 2003). Therefore, apparently either other *cis* elements outside the MTO1 region or additional *trans*-acting elements are involved in the regulation of the CGS transcript level.

An additional regulatory domain was indeed found recently within the N-terminal region of the AtCGS very close to the MTO1 domain (Hacham et al. 2006). This domain is found in another type of AtCGS mRNA that is identical to the AtCGS, but lacks 90 nt in its N-terminal region. Transgenic tobacco plants that overexpress this form of AtCGS lacking this domain show that this form is not subject to feedback regulation by methionine (Hacham et al. 2006), as reported for the full-length transcript (Onouchi et al. 2005). As a result, these transgenic plants accumulate twofold higher levels of methionine compared to plants expressing the full-length AtCGS, and about ninefold compared to wild-type plants (Hacham et al. 2006,

2008). A high level of methionine (up to sixfold compared to wild-type plants) was also found in tuber potatoes overexpressing this deleted form of AtCGS (Dancs et al. 2008).

The level of AtCGS is tightly regulated, as expected from the major role of methionine played in plant metabolism. In addition to the effect of SAM, which reduced the AtCGS transcript level (Onouchi et al. 2005), threonine in *Arabidopsis* (Avraham and Amir 2005) and the SAM-derived hormone ethylene in tomato (Katz et al. 2006) enhanced the transcript level of CGS. Other metabolites may also regulate the CGS level or activity, since it was recently shown that in cells starved of folates for a prolonged period, the regulatory N-terminal region of CGS was removed by proteolytic cleavage, leaving the enzyme active (Loizeau et al. 2007). The CGS expression level of potato was also found to up-regulate in light (Riedel et al. 1999), and we recently found that the expression of AtCGS was also regulated by sucrose mediated by light (R. Amir, unpublished results). Tight regulation of CGS is important, as was revealed from the severe morphological phenotype that usually appears in plants having lower or higher levels of CGS. Down-regulation of CGS in *Arabidopsis* led to severe growth retardation; these plants had reduced apical dominance and their floral organs were stacked. In addition, the chlorophyll content was lowered significantly (Gakiere et al. 2000a, b; Kim and Leustek 2000). Abnormal phenotype was also observed in *Arabidopsis*, potato and tobacco plants, overexpressing CGS and thus having higher methionine contents (Hacham et al. 2002; Kim et al. 2002; Dancs et al. 2008). However, this severe abnormal phenotype was not always detected, as was reported for alfalfa (Avraham et al. 2005), potato (Di et al. 2003; Kreft et al. 2003) and tobacco (Hacham et al. 2008).

In addition to the major role of CGS in the methionine biosynthesis pathway, it was recently suggested that CGS is a rate-limiting enzyme for selenium volatilization (Van Huysen et al. 2003). Transgenic Indian mustard overexpressing CGS showed two- to threefold higher selenium volatilization rates than wild-type plants when supplied with selenate or selenite. Furthermore, CGS seedlings were more tolerant to selenite than wild-type plants. These plants offer a promising approach for creating plants having an enhanced capacity to remove selenium from contaminated sites in the form of low-toxic volatile dimethylselenide (Van Huysen et al. 2003, 2004).

The regulatory role of threonine synthase

Several lines of evidence have shown that methionine biosynthesis is also regulated by threonine synthase (TS), the last enzyme of the threonine biosynthesis pathway in plants. This enzyme competes with CGS for their common substrate, *O*-phosphohomoserine (OPH) (Fig. 2) (Amir et al. 2002; Hesse et al. 2004). In vitro activity measurements indicate that TS in plants has a 250- to 500-fold higher affinity for OPH compared to CGS, causing reduced OPH availability for methionine synthesis (Ravanel et al. 1998; Curien et al. 1998). However, a modeling analysis performed in *Arabidopsis* suggested that TS and AtCGS have a similar kinetic efficiency for OPH, but OPH is used more by TS due to the higher concentration of this enzyme compared to AtCGS (Curien et al. 2003). The TS level in *Arabidopsis* was found to be sevenfold higher than the CGS level, causing the flux toward threonine synthesis to be fourfold higher than the flux toward methionine (Curien et al. 2003).

Analyses of transgenic and mutant plants have also shown that the ratio in protein levels between TS and CGS is important for determining the distribution of OPH between the two pathways (reviewed by Amir 2008; Hesse et al. 2004). For example, a reduction in TS levels using an antisense approach in potato and *Arabidopsis* plants caused a significant increase in methionine level (Zeh et al. 2001; Avraham and Amir 2005). However, if only the ratio between CGS and TS affects the partition of OPH between threonine and methionine biosynthesis, it is expected that when TS is reduced, the methionine level will increase proportionally and vice versa. However, antisense reduction in TS activity in potato and *Arabidopsis* plants caused a much stronger molar increase in methionine levels than the molar decrease in threonine levels (Avraham and Amir 2005; Zeh et al. 2001). In addition, transgenic tobacco, alfalfa and *Arabidopsis* plants overexpressing AtCGS had a significantly higher methionine level not accompanied by a reduction in threonine levels (Avraham et al. 2005; Hacham et al. 2002; Hacham et al. 2006). Moreover, *Arabidopsis* and potato plants expressing the antisense form of AtCGS showed a significant increase in OPH levels, while threonine increased only slightly (Gakiere et al. 2000a, b; Kreft et al. 2003). These results show that even when OPH is available for threonine synthesis, TS does not utilize it efficiently when the CGS level is low.

SAM, the first catabolic product of methionine, could be one of the factors controlling the flux toward threonine and methionine biosynthesis pathways. SAM negatively regulates the expression level of CGS (Onouchi et al. 2005) and positively regulates TS activity by an allosteric mechanism that increases the affinity of TS to OPH, as found by in vitro studies (Curien et al. 1998). This implies that methionine regulates its own synthesis through SAM. When a high level of methionine, and consequently SAM, is produced, the CGS level is down-regulated and TS activity is increased, causing reduced OPH availability for methionine synthesis (Curien et al. 1998). However, other evidence suggests that this scenario does not exist under in vivo conditions. Analyses of some mutants have

demonstrated that TS activity and threonine levels are not affected by SAM content. For example, it was found that in the *mto1* mutant where the CGS gene was mutated, free methionine increased 40-fold and SAM increased 3-fold, whereas the free threonine level was not altered compared to wild-type plants (Bartlem et al. 2000; Inba et al. 1994). In the *mto3* mutant having a mutation in the SAM synthase 3 gene, methionine increased more than 200-fold and SAM decreased by 35%, but the free threonine was again not affected (Shen et al. 2002). Moreover, in the *mmt* mutant, an opposite relationship prevailed between the level of SAM and threonine content, since this mutant has a higher level of SAM and a lower level of threonine (Kocsis et al. 2003). These results are intriguing, as they imply that SAM may not lead to the activation of TS in vivo.

Regulation of carbon/amino flow into methionine synthesis

The molecular and biochemical lines of evidence described above suggest that the endogenous level of OPH limits the levels of both methionine and threonine. To study this further, the endogenous *Arabidopsis* (Lee et al. 2005), as well as bacterial enzyme (Rinder et al. 2008) encoded to homoserine kinase (the enzyme that produced OPH), were overexpressed in *Arabidopsis* and potato plants, respectively. Expression of this enzyme does not lead to higher levels of methionine and threonine in these transgenic lines (Lee et al. 2005; Rinder et al. 2008), suggesting that homoserine kinase activity does not play a regulatory role in methionine and threonine biosynthesis pathways. However, when wild-type and transgenic potato and *Arabidopsis* plants overexpressing homoserine kinase were fed with homoserine, one metabolite upstream to OPH (Fig. 2), the levels of both amino acids increased significantly (Lee et al. 2005; Rinder et al. 2008). Moreover, a marked and significant increase in methionine content (a 180-fold increase above the level found in wild-type plants) was obtained when *Arabidopsis* plants overexpressing the AtCGS were fed with homoserine (Lee et al. 2005). These results suggest that under physiological conditions, the AtCGS and TS are substrate limited, and the levels of these amino acids are tightly regulated by the flux of the carbon/amino skeleton toward their synthesis pathways.

To test this assumption further, tobacco plants overexpressing AtCGS (Hacham et al. 2002) were crossed with those expressing bacterial feedback-insensitive aspartate kinase (AK) (Shaul and Galili 1992). Transgenic tobacco, *Arabidopsis* and alfalfa plants overexpressing this enzyme showed that the threonine level over-accumulated, while the methionine content, whose biosynthesis pathway diverged from this branch, was not significantly changed (Shaul and Galili 1992; Ben Tzvi-Tzchori et al. 1996; Galili et al. 2000). Since threonine accumulates in these plants, it is expected that the levels of intermediate metabolites of its pathway will also increase, including OPH. Therefore, expressing these two genes could lead to a higher methionine level compared to plants overexpressing the AtCGS alone. Plants co-expressing these two genes, indeed, have significantly higher methionine and threonine levels compared to levels found in wild-type plants, but the methionine level does not increase beyond that found in plants expressing only AtCGS (Hacham et al. 2008). This result could be explained by the feedback inhibition regulation mediated by SAM on the transcript level of AtCGS when methionine increases beyond a certain threshold (Chiba et al. 2003). To test this assumption, plants expressing the bacterial AK were crossed with plants expressing mutated forms of AtCGS in which the N-terminal region of AtCGS, or the 90 nt-domain, was deleted. These two forms of AtCGS are methionine/SAM insensitive. In the newly produced plants expressing the mutated forms of AtCGS and AK, the methionine level was significantly increased compared to plants expressing only these forms of AtCGS (about 4.5-fold higher). However, when compared to wild-type plants, methionine increased and was about 110- to190-fold higher. Threonine levels doubled in these plants compared to wild-type plants (Hacham et al. 2008). These results suggest that the carbon/amino skeleton limits methionine synthesis, but this can hardly be seen under normal physiological conditions, since the regulatory role of SAM on the expression level of AtCGS has a stronger effect. The results obtained in this study also suggest new ways of producing transgenic crop plants containing increased levels of methionine and threonine (an important essential amino acid that often limits the nutritional value of cereals) and hence having improved nutritional quality.

The regulatory role of cysteine, the sulfur donor, in the methionine biosynthesis pathway

To determine if cysteine content limits methionine synthesis, methionine content was analyzed in transgenic tobacco plants having higher levels of cysteine due to overexpression of the yeast *Met25* gene encoded to the last enzyme of the cysteine biosynthesis pathway, *O*-acetylserine (thiol) lyase (yOASTL). These plants showed that the methionine level did not change significantly (Matityahu et al. 2005). However, overexpressing this yeast gene in transgenic flax plants resulted in the methionine level increasing three- to eightfold compared to the levels of non-transgenic flax (Czuj et al. 2009). In these transgenic plants, the level of the cysteine metabolite, glutathione, increased significantly. Higher levels of glutathione were also observed in many other transgenic plants having high levels of cysteine (e.g., Saito et al. 1994; Blaszczyk et al.

1999; Noji et al. 2001; Nikiforova et al. 2002; Wirtz and Hell 2003; Stiller et al. 2007). This suggests that cysteine is channeled toward glutathionine synthesis, whereas the ability of high cysteine content to affect methionine content depends on the plant species.

The observation that transgenic plants overexpressing the truncated form of AtCGS, and thus having higher levels of methionine, emitting significantly higher levels of sulfur-containing metabolites such as methanethiol, dimethylsulfide and carbon disulfide (Hacham et al. 2002; Boerjan et al. 1994), suggest that sulfur content as well as cysteine do not limit methionine synthesis in plants. To further test this assumption, we crossed plants overexpressing yeast OASTL targeted to the chloroplast (Matityahu et al. 2005) with those overexpressing the AtCGS (which is naturally active in the chloroplasts) (Hacham et al. 2006). Plants overexpressing both genes showed slightly, but significantly, higher amounts of methionine and the methionine metabolite, SMM, compared to plants overexpressing the AtCGS alone (R. Amir, unpublished results). The results suggested that higher levels of cysteine in the chloroplasts could contribute to methionine synthesis when a high expression level of CGS exists. This level most probably cannot increase beyond a certain threshold due to the regulation of SAM on CGS transcript level (Chiba et al. 1999).

The relationship between sulfate availability and methionine content in plants is not yet clear. It was recently found that over a broad time range of sulfate deprivation, methionine levels do not change significantly (Nikiforova et al. 2005, 2006). The plants were able to keep the methionine level constant, which is always low in plants. The level of SAM, however, was depleted under these conditions, while the levels of threonine and isolecuine (which compete with methionine for the carbon/amino skeleton) increased. These results suggest that factors other than sulfur content are involved in methionine homoeostasis in plants during sulfur starvation. One factor that might assist in maintaining the methionine homoeostasis in plants could be the methionine-recycled pathways that recycle the methionine moieties to regenerate methionine (described below). Indeed, it was found that the transcript levels of SAM synthase and adenosylhomocysteine hydrolase, two enzymes involved in recycling methionine moieties, are induced during sulfur starvation (Nikiforova et al. 2005).

The regulatory role of methyl flow in methionine synthesis

Methionine synthase, the last enzyme of the methionine biosynthesis pathway, catalyzes the methylation of homocysteine to methionine with 5-methyltetrahydrofolate as a methyl group donor. This enzyme is also involved in the massive turnover of methionine in methyl transfer reactions through SAM and the methyl cycle (Figs. 1a, 2). The methyl transfer reactions and methionine synthase are localized in the cytosol (Eichel et al. 1995; Eckermann et al. 2000). Thus, it was suggested that homocysteine was produced in the chloroplasts by cystathionine β-lyase transfer from this compartment to the cytosol. However, recent studies have shown that three forms of methionine synthase are found in *Arabidopsis*, one of which is present in the chloroplasts, suggesting that methionine can also be produced in the chloroplasts to support protein synthesis and methionine metabolism inside the chloroplast (Ravanel et al. 2004). Further studies are required to reveal whether the methionine synthesized in the chloroplast is exported to the cytosol, thus contributing to methionine metabolism in this compartment.

As expected from its massive role in methionine recycling pathways, methionine synthase does not play any regulatory or limiting role in methionine biosynthesis, as suggested by overexpressing the gene encoding this enzyme in potato plants. Although the level of this enzyme increases significantly, methionine content does not change significantly (Zeh et al. 2002; Hesse and Hofgen 2003).

The regulatory roles of lysine and threonine in determining methionine levels in plants

Two major branches exist in the aspartate family of amino acids. One leads to the lysine biosynthesis pathway, while the other leads to threonine, methionine and isoleucine synthesis (Fig. 2). Regulation of the carbon/amino skeleton flux within these two branches is quite complicated, and metabolites from one branch affect flux toward the other branch (Azevedo et al. 2006; Jander and Joshi 2010). Application of both threonine and lysine leads to methionine starvation, since they both inhibit AK activity (Lee et al. 2005) (Fig. 1). However, it was recently found that threonine and lysine, when applied separately, led to methionine accumulation. Using transgenic plants and feeding experiments, it was found that high levels of threonine enhance the expression levels of AtCGS and thus led to an increase in methionine content (Avraham and Amir 2005; Hacham et al. 2008). However, the nature of this regulation is not yet clear, since studies using the in vitro transcription/translation system suggest that threonine does not directly affect the level of AtCGS (Hacham et al. 2008).

In addition to threonine, previous studies have also shown a positive correlation between methionine and lysine content in plants. Transgenic barley plants that constitutively express the feedback-insensitive bacterial

dihydrodipicolinate synthase (bDHPS), the first unique enzyme of lysine biosynthesis pathway, exhibited a 14-fold increase in free lysine and an 8-fold increase in free methionine (Brinch-Pedersen et al. 1996). In addition, seeds of three sets of *Arabidopsis* transgenic plants exhibiting significantly higher levels of lysine resulting from the seed-specific expression of bDHPS and RNAi of lysine-ketoglutarate reductase/saccharopine dehydrogenase, the catabolic enzyme of lysine, showed a significant 80-fold increase in lysine content and 51-fold increase in methionine level compared to wild-type seeds (Zhu and Galili 2003, 2004). These results suggest that a higher level of lysine enhances the production of methionine or reduces its catabolism.

To elucidate the relationship between these two amino acids and to study the factors regulating methionine synthesis, transgenic tobacco plants overexpressing AtCGS that exhibit higher levels of methionine were crossed with those overexpressing bDHPS having a significantly higher level of lysine (Shaul and Galili 1992). Methionine and SMM levels were found to be significantly elevated in plants co-expressing both transgenes compared to those expressing only AtCGS; the level of lysine remained the same as that overexpressing only bDHPS (Hacham et al. 2007). It was found that a high level of lysine down-regulates the transcript expression level of SAMS. This led to a reduction in the amount of SAM, which negatively regulates the level of AtCGS transcript (Chiba et al. 2003). As a result, the expression level of AtCGS increased and consequently the level of methionine (Fig. 2, dotted line) (Hacham et al. 2007). Methionine level can also be enhanced by a reduction in flux toward SAM and its metabolites (Giovanelli et al. 1985).

The results described above imply that the two methionine "brothers" in the aspartate family, threonine and lysine, that their biosynthesis pathways compete with the methionine pathway for the carbon/amino skeleton, regulate methionine level, keeping its level high. If this indeed occurs in plants, it insures that if a mutation occurs or conditions leading to higher levels of threonine or lysine exist, the methionine level will not be significantly affected. Regulating the methionine level is important due to the major role that methionine plays in protein synthesis and plant metabolism.

From a biotechnological point of view, the results described here show that significantly higher methionine contents can appear with significantly higher levels of threonine or lysine. The latter two are essential amino acids that often limit the nutritional value of cereal grains where a low level of methionine is also found (Galili et al. 2005). However, the manipulation of AtCGS and bDHPS in the vegetative tissues of crop plants should be considered carefully, since the level of SAM in these plants is significantly reduced, which might affect the level of essential methionine/SAM metabolites. A reduction in SAM content can lead, for example, to lower lignin content (as described above for the *mto3* mutants) (Shen et al. 2002). A reduction in lignin content in plant tissues may sometimes be beneficial, since it is important to reduce lignin content in plants used to feed ruminant animals that are unable to digest lignin (Deschamps et al. 1996), as well as for biofuel production (Sticklen 2008). However, manipulation of AtCGS and bDHPS could be considered in seeds, since a different fate for methionine is more likely in vegetative tissue than in seed tissue. Seeds are committed to the synthesis and accumulation of seed-storage proteins; hence, a lower catabolism of methionine via SAM is expected in this tissue, and more of the methionine should be incorporated into the seed-storage proteins.

The regulatory role of methionine catabolic pathways

The role of SAMS in controlling methionine content

The results described above show that lysine regulates the transcript level of SAMS and thus controls the methionine level. This result linked previous evidence showing that SAMS plays a major role in controlling methionine levels in plants. The overexpression of SAMS in *Arabidopsis* does not lead to altered levels of methionine and SMM, although CGS activity is reduced in this mutant (Kim et al. 2002). This reduction can be explained by the regulatory role of SAM in the transcript level of CGS, as shown by the Naito group (Onouchi et al. 2005; Haraguchi et al. 2008; Onouchi et al. 2008). As expected, a reduction in the expression level of SAMS leads to significantly higher levels of methionine in plants, as shown by several mutants and transgenic plants. For example, the *Arabidopsis* mutant (*mto3*) exhibiting reduced SAMS3 activity had an over 200-fold methionine content compared to wild-type plants (Goto et al. 2002; Shen et al. 2002). This high level of methionine was associated with a low level of SAM and, as a result, the lignin content, one of the major metabolic sinks for SAM, decreased by 22% compared to wild-type plants (Shen et al. 2002). A relationship between the level of SAMS and lignin content was also observed in tomato plants, in which the level of SAMS increased significantly under salt stress (Sánchez-Aguayo et al. 2004). A more severe phenotype appeared in SAMS silencing *Arabidopsis* plants. In these plants, the level of methionine increased 250-fold compared to wild-type plants, but severe abnormalities were observed (Kim et al. 2002). A severe abnormal phenotype also appeared in tobacco plants where the SAMS level was reduced significantly (Boerjan et al. 1994). This suggests that a reduction in the expression level

of SAMS could significantly increase the level of methionine, but due to the importance of SAM in plant metabolism, such a reduction would lead to severe abnormal phenotypes. Alternatively, high levels of methionine can be toxic to the plants.

The regulatory roles of methionine recycling pathways in methionine content

Three recycling pathways can regenerate the moieties comprising methionine following the formation of SAM metabolites (Fig. 1a): (1) the methyl cycle, in which the methyl group of methionine/SAM is donated to methyl transfer reactions. This leaves *S*-adenosylhomocysteine, which recycles to methionine through homocysteine. The cytosolic form of methionine synthase combines the methyl group to homocysteine to regenerate methionine (Roje 2006); (2) the SMM cycle, which operates by the activities of SAM-Met *S*-methyltransferase (MMT) and homocysteine *S*-methyltransferase (HMT) (Ranocha et al. 2001); and (3) the Yang cycle (also termed the MTA cycle), where ethylene, biotin and polyamines are synthesized, and the methylthio moieties are recycled to methionine via methylthiobutyrate (Fig. 1a) (Yang et al. 1990).

Although the importance of these pathways is accepted, little is known about their regulation and contribution to the methionine pool. In addition, the nature of the competition for methionine between protein synthesis and SAM synthesis in plants has not yet been clarified fully. Studies on the metabolic fates of methionine using the aquatic plant *Lemna paucicostana* indicated that the synthesis and turnover of SAM accounts for 80% of the methionine metabolism, whereas the synthesis of proteins drives about 20% of the methionine metabolism (Giovanelli 1987). In mature *Arabidopsis* rosette leaves, however, Ranocha et al. (2001), which use radioactive methionine and in silico modeling, about half of the soluble methionine converts to SAM and SMM, and half to protein synthesis (Ranocha et al. 2001). This also suggests that the combined rate of release of methionine from the storage pool and from protein turnover is similar in magnitude to SAM synthesis (Ranocha et al. 2001). A quantitative analysis of the methionine metabolism in *Lemna*, which does not produce ethylene, showed that the Yang cycle (used for polyamine synthesis) accounts for 6% of the methionine, whereas the de novo synthesis of methionine contributed 19%, and recycling from SAM-dependent methylations accounted for 75% of the methionine pool (Giovanelli et al. 1985). It was also suggested that SAM was used predominantly for transmethylation, the major product of which was phosphatidylcholine, with progressively small amounts directed to the synthesis of methyl groups of pectin and chlorophyll methyl esters (Mudd and Datko 1986). The role of the methyl cycle in methionine content is far from being resolved and further studies are required to reveal it.

Recent studies performed in flowers of *Nicotiana suaveolens* showed that higher SAM levels were measured in the evening and at night, which corresponded to the time when the major floral scent compound, methyl benzoate, was synthesized by a SAM-dependent methyl-transferase and when this enzyme exhibited its highest activity. Transcript accumulation patterns of both SAMS and methionine synthase, two enzymes involved in the synthesis and regeneration of SAM, perfectly matched those of methyl-transferase (Roeder et al. 2009). This suggests that in different organs and at different times, SAM can be converted to preference compounds to produce different metabolites.

The role of the Yang cycle in methionine homoeostasis is especially interesting in plants that produce ethylene and in climacteric fruits where bursts of ethylene occur during the ripening stage. To further study the role of the Yang cycle in SAM production and its homoeostasis, Bürstenbinder et al. (2007) used an *mtk* mutant that has a disruption of the Yang cycle. Based on their data, the researchers conclude that the Yang cycle contributes to SAM homeostasis, especially when de novo SAM synthesis is limited, such as at sulfur starvation (Nikiforova et al. 2005, 2006). The data also showed that this cycle was required to sustain a high level of ethylene synthesis. However, additional evidence suggests that in addition to recycling the methionine moieties via the Yang cycle, the de novo synthesis of methionine is required when high rates of ethylene production are induced (Katz et al. 2006). This was suggested based on the observation that the transcript expression level of CGS was positively correlated to ethylene production during the ethylene burst of the climacteric ripening of tomato fruit and during leaf wounding on ethylene emission (Katz et al. 2006). The level of methionine also increased accordingly under these conditions (Katz et al. 2006).

The role of the third cycle, the SMM cycle, which is unique to plants, is also unclear. Number roles were proposed for this cycle in plants, including the following: (1) SMM mediates the long-distance transport of labile methyl moieties and reduces sulfur (Giovanelli et al. 1985); (2) SMM functions as a storage form of methionine to protein synthesis and keeps the free methionine pool from being depleted by an overshoot in SAM synthesis (Pimenta et al. 1998); and (3) SMM controls the SAM level in plant tissue. Using radioactive tracer measurements, Ranocha et al. (2001) suggested that the cycle consumes half the SAM produced, and that the cycle serves to stop the accumulation of SAM rather than prevent the depletion of free methionine. Controlling the level of SAM is crucial to many methyl transfer reactions, since the ratio between

SAM and the *S*-adenosyl-homocysteine metabolite in the methyl cycle, which is a potent inhibitor of methyl-transferases, determined the activity of these essential enzymes (Chiang et al. 1996). This assumption was recently strengthened by the observation that the *Arabidopsis mmt* mutant, which lacks the SMM cycle, had a significantly higher level of SAM and a lower level of *S*-adenosylhomocysteine than wild-type plants and, consequently, a higher methylation ratio (SAM to *S*-adenosylhomocysteine ratio). These results also support the hypothesis that the SMM cycle contributes to the regulation of SAM levels, as suggested by Kocsis et al. (2003). Further support came recently from studies showing that feeding with methionine affected SAM level, which increased tenfold, but mainly contributed to the SMM level that increased, followed by a steady value of SAM (Rebeille et al. 2006). The role of the SMM cycle is yet to be revealed, since not all plant species (such as potato) have been shown to contain or use this cycle.

Methionine catabolism: the role of methionine γ-lyase

It was suggested that SMM functions as a storage or transport form of methionine when the level of this amino acid is present in excess (Bourgis et al. 1999; Kocsis et al. 2003). The accumulation of SMM, however, cannot deal with a large excess of methionine, possibly because of the energy cost of its synthesis (one ATP per SMM molecule) (Mudd and Datko 1986). Therefore, methionine must be removed through other catabolic pathways.

Several lines of evidence suggest that methionine can be degraded in plants. These include findings that plants having a higher methionine content emit volatile compounds containing sulfur and methyl moieties, such as methanthiol, dimethyl disulfide or dimethyl sulfide, which suggests that these metabolites are methionine degradation products (Hacham et al. 2002; Boerjan et al. 1994). The enzymes and pathways responsible for the production of these volatiles and their physiological significance are still unknown. Recently, a gene encoded for methionine γ-lyase was cloned from the *Arabidopsis* genome (Rebeille et al. 2006; Goyer et al. 2007). This enzyme catalyzes methionine into methanethiol, α-ketobutyrate and ammonia. The researchers found that this cytosolic enzyme is abundant in all plant organs, except in the seeds. Western blot studies indicated that this gene was expressed under standard growth conditions and was strongly induced when the cells accumulated methionine (Rebeille et al. 2006). The enzyme has a relatively high K_m level for methionine (~10 mM), indicating that this pathway operates preferentially when methionine has accumulated above a certain value in the cytoplasm. Under sulfate limitation, but not under normal growth conditions, knockout of the single *Arabidopsis* methionine γ-lyase gene significantly increased methionine content (ninefold) and SMM content in leaf (Goyer et al. 2007). This finding suggests that this catabolic pathway plays a role during sulfate starvation, but since the level of methionine is not altered under this stress (Nikiforova et al. 2005), the situation is unclear and further studies are required to reveal the role of this enzyme.

Questions still remain regarding the role of methanthiol, the catabolic product of methionine, in plant metabolism. Rebeille et al. (2006) suggest that 75% is released from cells as a volatile compound and 25% react with *O*-acetylserine (the metabolite of the cysteine biosynthesis pathway) to produce *S*-methylcysteine. However, the authors could not identify metabolites of *S*-methylcysteine and suggest that this compound, which is produced in the cytoplasm, is transferred rapidly to the vacuole and could play a storage role. However, based on their observations, Goyer et al. (2007) suggest that under low-sulfur content, about 9% of radioactive methionine appears as cysteine in the proteins and that methanthiol can convert to cysteine. They therefore assume that the methanthiol pathway is an alternative to the reverse transsulfuration pathway operating in certain bacteria (Saint-Girons et al. 1988) in which methionine converts to cysteine. It was recently found that the second catabolic product of methionine γ-lyase, α-ketobutyrate, produced in the cytosol could be transported to plastids and integrated into the isoleucine biosynthesis pathway (Joshi and Jander 2009). A relationship between high methionine and isoleucine contents was also reported in potato, tobacco and *Arabidopsis* plants (Zeh et al. 2001; Dancs et al. 2008; Hacham et al. 2008; Rebeille et al. 2006). It was recently revealed that isoleucine biosynthesis, through the activity of methionine γ-lyase, plays a significant role under abiotic stresses, such as osmotic, drought and salt stresses (Less and Galili 2008; Joshi and Jander 2009; Joshi et al. 2010). The reasons for this are not yet clear.

Methionine catabolism: the role of enzymes leading to glucosinolates synthesis

In addition to the role of methionine γ-lyase in methionine catabolism, it was found that methionine also catabolized by methyl thioalkylmalate synthases (MAM) to produce glucosinolates (GSLs), a group of plant secondary metabolites that exhibit repellent activity against herbivore insects and pathogens. These compounds are produced in plants of Brassicaceae and other related families from methionine or from aromatic amino acids and can represent up to 30% of the total sulfur content of plant organs (Hirai et al. 2007). Methionine-derived GSLs with diverse side chains of various lengths are the major GSLs in *Arabidopsis*. It was recently reported that in the knockout lines of the *atleuc1-1*

gene, one of the enzymes of the methionine-derived GSLs synthesis pathway, the levels of six methionine-related metabolites, SAM, methionine, SMM, methionine sulfoxide, *O*-acetylserine and 5′-deoxy-5′-methylthioadenosine, significantly increased ($P < 0.05$) compared to wild-type plants. The latter metabolite, 5′-deoxy-5′-methylthioadenosine, is synthesized as a byproduct in the biosynthesis of spermine and spermidine from decarboxylated *S*-adenosylmethionine, and in the biosynthesis of nicotianamine and ethylene from *S*-adenosylmethionine (Yang and Hoffman 1984). This result suggests that when the metabolic flow from methionine to methionine GSLs biosynthesis is blocked, redirection to the primary methionine metabolism occurs. The increase in *O*-acetylserine may also indicate some changes in sulfur status in the cell. Notably, the knockout of MAM does not show significant changes in the methionine-related metabolites (Sawada et al. 2009).

As expected from the high sulfur level in GSLs originating from methionine (at least three sulfur atoms per molecule), the level of GSLs was reduced under sulfur deficiency. It was found that the transcription of many genes in glucosinolate biosynthesis, including MAM and aminotransferases, which are the main methionine chain elongation enzymes leading to GSLs synthesis (Kroymann et al. 2001; Textor et al. 2007), were down-regulated under sulfur starvation (Hirai and Saito 2004; Nikiforova et al. 2005). In the meantime, genes encoding myrosinases, sulfate transporters and nitrilases were up-regulated (Kutz et al. 2002; Maruyama-Nakashita et al. 2006). Myrosinases is the enzyme that, upon tissue damage, hydrolyzes the GSLs to produce a variety of degradation products, typically isothiocyanates, thiocyanates and nitriles, which have a wide range of biological effects. These changes allow a reduction in sulfur assimilation into GSLs and a remobilization of sulfur in GSLs to produce sustainable amounts of sulfur source for protein synthesis and essential functions other than defense. Sulfur fertilization, however, enhances the level of GSLs and the resistance to *Brassica napus* against the fungal pathogen (Bloem et al. 2007).

It was recently found that the expression level of MAM was coordinately expressed with other genes of methionine metabolism under various growth conditions in *Arabidopsis* plants. Together with AK, CGS and the genes involved in the catabolism of methionine via SAM, this gene is expressed into multiple growth-associated metabolites (Less and Galili 2009), suggesting that GSLs synthesis coordinates with methionine synthesis and its catabolism in plants. Moreover, by using a bioinformatics analysis of public microarray data of *Arabidopsis*, these researchers also reported that the transcription of the catabolic genes of methionine (SAMS, methionine γ-lyase and MAM) were primarily more sensitive to fluctuation in stress-associated signals than biosynthesis genes (Less and Galili 2008). This means that their expression levels increased or decreased shortly after the stress appeared, modulating the levels of methionine and its metabolites (Less and Galili 2008).

Toward improving methionine content in vegetative tissues in plants for enhanced nutritional quality

The importance of methionine in human and livestock nutrition

Mammals do not produce methionine de novo and require the intake of this sulfur-containing amino acid from dietary sources. Therefore, methionine belongs to essential amino acids. Cysteine is considered a "conditionally" essential amino acid, since under most circumstances, the metabolism of methionine in the body can provide cysteine in necessary amounts. Plants producing both amino acids are the ultimate source of these in the food chain of animals and humans. Cysteine and methionine are required for protein synthesis, in which they are nearly always directly involved regarding the catalytic, structural or electrochemical functions of proteins, and give rise to key enzymatic cofactors (Saito 2000). In addition to their role in protein synthesis, the sulfur amino acids are the primary route for the body to incorporate sulfur into a vast array of molecules. They serve as metabolic precursors in the production of glycosamineglycans, an important component in connective tissues such as cartilage and skin (Jez 2008). Likewise, the synthesis of vitamins containing sulfur, such as thiamine, biotin and Co-enzyme A, depends on the availability of these amino acids. The metabolism of these amino acids also links into pathways involved in glutathione (an important antioxidant and xenobiotic detoxification molecule), SAM, the universal methyl donor, and folic acid.

Since methionine is one of the four main dietary sources of methyl groups, its deficiency can be associated with methylation-related disorders, such as fatty liver, atherosclerosis, neurological disorders and tumorigenesis (Poirier 2002; Fukagawa 2006; Fukagawa and Galbraith 2004). Methionine through SAM also influences DNA synthesis and the expression of genes; its deficiency has been associated with DNA fragmentation and strand breaks (Lertratanangkoon et al. 1996). Chronic lack of methionine and folic acid decrease the body's threshold to handle chemical toxicity and may be related to the development of colorectal and breast cancer (Jez 2008). In animals, methionine depletion lowers the threshold of chemical-induced toxicity, suggesting that this may be significant in carcinogenesis processes (Lertratanangkoon et al. 1996).

Although methionine is important for animal and human nutrition, it belongs to those essential amino acids that are often found in low contents in plants, thus limiting the

nutritional quality of protein for humans and livestock worldwide (Galili et al. 2005; Tabe and Higgins 1998). When one amino acid is found at a low level, the other amino acids are catabolized and used as an energy source. Among these nutritional-limiting essential amino acids, methionine is found at a low level in most crop plants, but is particularly deficient in legume plants (e.g., soybean, pea, bean, chickpea, alfalfa, lentil, clover), which are among the most important nutritional sources of protein for humans and livestock (Galili et al. 2005). The biological value of a plant-based diet with limited methionine content can be equivalent to only 50–75% of that of a diet with balanced, essential amino acids. In cultures having a primarily vegetarian diet or in developing countries in which plant-derived foods are predominant, this can lead to non-specific signs of protein deficiencies in humans, such as lowered resistance to disease, decreased blood proteins, and retarded mental and physical development in young children. This syndrome is referred to as protein–energy malnutrition (PEM); the World Health Organization (WHO) estimates that around 30% of populations in the developing world suffer from PEM.

Methionine levels are generally not limited in human foods in Western countries due to the significant consumption of livestock products, meat, eggs and milk, which generally contain adequate levels of this essential amino acid. However, methionine deficiencies in plant-derived feeds for farm animals limit both animal growth and products, such as wool growth in sheep, milk production by dairy animals and meat quality (Pickering and Reis 1993; Tabe and Higgins 1998; Xu et al. 1998). To meet the requirements of monogastric animal diets, methionine was recently added in the synthetic form to an animal-based diet in many Western countries. Furthermore, it was also added to process soybean products (e.g., soybean milk and tofu) for human consumption. For ruminant animals, however, methionine must be supplied in the form of proteins that are resistant to rumen proteolysis, since unprotected dietary proteins are rapidly degraded by bacteria in the rumen and converted to bacterial proteins. For this reason, the sulfur-rich protein candidate for expression in transgenic forage legumes should be resistant to proteolytic degradation in the rumen (Bagga et al. 2004).

Increasing methionine content in forage crops and pasture plants

Due to the importance of methionine in human food and animal feed, many efforts have been made to produce plants having higher methionine content. However, traditional plant breeding methods have been unsuccessful in increasing the level of sulfur amino acids. Two main strategies have been used to increase methionine content via gene transfer technology: manipulation of the methionine biosynthetic pathway and the creation of additional protein "sinks" for methionine storage (reviewed by Amir and Tabe 2006). Both molecular approaches have provided new insights into the control of methionine level in plants, and in some cases have yielded significantly higher levels of methionine and consequently improved plant nutritional value (described below).

Efforts have been made to increase the methionine content and thus improve the quality of vegetative tissues, with particular attention to forage legumes. In the late 1980s and during the 1990s, researchers assumed that the level of methionine in vegetative tissues was sufficient to support the methionine metabolism and synthesis of additional methionine-rich proteins. Therefore, they used genes encoding methionine-rich, seed-storage proteins fused to a constitutive promoter and expressed them in various plants. However, in vegetative tissues, these proteins were found to be less stable than in seeds. For example, Brazil nut 2S albumin constitutively expressed in *Vicia narbonensis* and tobacco plants do not accumulate in the vacuoles of mesophyll leaf cells (Bagga et al. 1995). This is most probably because in seeds they can accumulate in protein bodies derived from the endoplasmic reticulum (ER) or in storage vacuole-derived protein bodies, which may protect them from proteolysis. Whereas ER-accumulating storage proteins are retained in the ER of vegetative cells, vacuolar proteins targeted to vacuoles may enter the protease-rich vegetative vacuole and risk being degraded. To test this assumption, an ER retention signal (KDEL) was combined with the pea vicillin storage protein, a vacuolar protein. As a result, the vicillin stays within the ER, stabilizing it 100 times more than unmodified vicillin in the leaves of transgenic plants (Wandelt et al. 1992).

Consistent with these findings are those obtained in transgenic plants overexpressing β-zein, γ-zein and/or δ-zein, which have an ER retention signal. These methionine-rich storage proteins originating from maize accumulate naturally in seed ER-derived protein bodies (e.g., Bagga et al. 2004; Bellucci et al. 2005). Notably, tobacco and alfalfa plants overexpressing β-zein and δ-zein produced novel ER-derived protein bodies in leaves that apparently protect them from degradation (Bagga et al. 1995, 2004). Co-expressing these two proteins together significantly increased the level of δ-zein (Bagga et al. 1995, 2004), implying that interactions between different zeins are important for their accumulation. Mainieri et al. (2004) took this knowledge further by designing a chimeric protein composed of phaseolin, the major seed protein of bean, and 89 amino acids of γ-zein, which has the ER retention signal. Unlike wild-type phaseolin, the chimeric protein, which they called "zeolin", accumulates to very high amounts in the leaves of transgenic tobacco. The

researchers conclude that the γ-zein portion is sufficient to induce the formation of protein bodies also when fused to another protein. Expression of this protein in other organelles, such as chloroplasts, led to an unstable protein, demonstrating the role of the ER in protecting the forging protein (Bellucci et al. 2005). Since the storage proteins of cereals and legumes nutritionally complement one other, zeolin can be used as a starting point for such manipulations in the future (Mainieri et al. 2004).

Expression of methionine-rich storage proteins in leaves shows, as found in some of the transgenic seeds, that the elevation of foreign proteins was at the expense of the other sulfur-rich endogenous proteins, as well as other sulfur compounds in the cells. This indicates that methionine availability limits the synthesis of methionine-rich proteins. It was therefore suggested that in addition to expressing methionine-rich storage proteins, it is desirable to increase the soluble methionine content by manipulating the methionine biosynthesis pathway or its catabolism (Tabe and Droux 2001; Amir 2008). Several approaches have been tested, including overexpression of CGS, lower expression levels of TS and SAMS, and combinations of AK and CGS, and of CGS and bDHPS, as described (also reviewed by Galili et al. 2005; Amir and Tabe 2006; Amir 2008). To further test if the level of soluble methionine indeed limits the production of sulfur-rich proteins in vegetative tissues, transgenic alfalfa plants constitutively expressing β-zein were fed with methionine. It was found that the level of β-zein increased significantly (Golan et al. 2005). This finding encouraged the researchers to cross between transgenic alfalfa plants constitutively expressing β-zein and those constitutively expressing AtCGS that exhibit significantly higher levels of methionine (Avraham et al. 2005). Compared to plants expressing only β-zein, both those co-expressing transgenes showed significantly enhanced levels of β-zein concurrently with a reduction in the level of soluble methionine when compared to plants expressing only AtCGS. This implies that more soluble methionine was incorporated into the β-zein in the crossed plants (Bagga et al. 2005; Golan et al. 2005). The increase in β-zein in alfalfa plants is of particular nutritional importance to ruminant animals, as this protein is resistant to rumen proteolysis (Bagga et al. 2004). These results further suggested that to enhance the nutritional quality of forage plants by increasing their methionine content in proteins, one should express both an enzyme such as CGS that leads to high methionine synthesis and methionine-rich storage proteins in the same plant tissues (Golan et al. 2005).

Although these results are encouraging, a recent study conducted in transgenic potatoes has shown that this strategy of combining the methionine-rich protein of β-zein with that of the deleted form of AtCGS, although leading to a sixfold increase in methionine and β-zein, showed a phenotypically abnormal phenotype. These transgenic plants exhibited severe growth retardation, changes in leaf architecture and a 40–60% reduction in tuber yield. Furthermore, the color of the transgenic tubers was altered due to the reduced amounts of anthocyanin pigments (Dancs et al. 2008). Therefore, additional studies are required to obtain crop plants having a higher methionine content with low morphological phenotype. Morphologic phenotype is less expected in seeds where the methionine metabolism is supposed to differ from vegetative tissues. However, knowledge about methionine synthesis and its metabolism in seeds is still limited and requires further study (Amir 2008; Lee et al. 2008). We believe that the knowledge accumulated of vegetative tissues described in this review will help in manipulating the methionine metabolism in seeds and thus help produce seed crop plants having higher levels of methionine and, consequently, higher nutritional quality.

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