Towards Improving Methionine Content in Plants for Enhanced Nutritional Quality

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ABSTRACT

Methionine is a nutritionally essential sulfur-containing amino acid whose low level in plants diminishes their value as a source of dietary protein for humans and animals. Methionine is also a fundamental metabolite in plant cells since, through its first metabolite S-adenosylmethionine (SAM), it controls the levels 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 its synthesis and catabolism in plants are not fully known. In recent years, genetic molecular biology techniques have been used to increase and decrease the expression levels of several genes encoded to enzymes in the methionine metabolism in order to gain more knowledge about its role in plant metabolism, as well as to increase methionine level and thus improve the nutritional quality of plants. In this review, recent progress made in the molecular characterization of these genes is summarized, and specific examples are given of the regulation of metabolic pathways required for a tailor-made improvement of methionine content, with minimal interference on plant growth, phenotype and productivity. Several different manipulations of methionine biosynthesis and metabolism pathways, in addition to the expression of methionine-rich storage proteins and their effects on plant methionine content, are described. The studies have resulted in the identification of steps important for the regulation of flux through the pathways and for the production of transgenic plants having increased free and protein-bound methionine. These molecular approaches have provided new insights into the control of methionine level in plants, and in many cases, have resulted in significant improvements in the nutritional value of plants.

Keywords: cystathionine γ-synthase; methionine metabolism; methionine rich storage proteins; nutritional improvement; regulation; S-adenosylmethionine

Abbreviations: AtCGS, Arabidopsis CGS; AK, aspartate kinase; CGS, cystathionine γ-synthase; DHPS, dihydrodipicolinate synthase; HMT, homocysteine S-methyltransferase; MMT, methionine methyltransferase; OASTL, O-acetyl(thiol)lyase; OPH, O-phosphohomoserine; SAM, S-adenosylmethionine; SMM, S-methylmethionine; TS, threonine synthase

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THE IMPORTANCE OF METHIONINE IN HUMAN AND LIVESTOCK NUTRITION

Humans and livestock cannot synthesize nine out of the 20 amino acids used as the building blocks of their proteins and must obtain these so called “essential amino acids” from their diets. The nutritional value of plants, which are the world’s most dominant sources of human food and animal feed, is predominantly inadequate due to their limited content of some essential amino acids. Cereal grains are particularly limited in the levels of lysine, tryptophan and threonine, while legumes (e.g., soybean, pea, bean, chickpea, alfalfa, lentil, clover) are mainly limited in the contents of sulfur amino acids, methionine and cysteine (Tabe and
Higgins 1998; Galili et al. 2005). The biological value of a plant-based diet with limited methionine content can be equivalent to only 50% to 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 derived foods are predominant, this can lead to non-specific resistance to disease, decreased blood proteins and retarded growth and development in young children (Waterlow et al. 1975). This syndrome is referred to as Protein-Energy Malnutrition. The World Health Organization estimates that around 30% of the populations in the developing world suffer from Protein-Energy Malnutrition. In addition, because methionine is one of the four main dietary sources of sulfur-containing peptides, and the majority of them are produced by bacteria in the rumen, such as reduced wool growth in sheep, milk protein content in milk, and egg production in laying hens (Kang and Clarke 1994; Roje 2006). Hence, as a donor for methyl groups, methionine through SAM regulates essential cellular processes such as cell division, synthesis of cell wall, synthesis of chlorophyll and membrane synthesis (Roje 2006). In higher plants, SAM is also the precursor for the hormone ethylene, which regulates both developmental stages including the ripening of fruit and senescence (Yang and Hoffman 1984; Miyazaki and Yang 1987; Yang et al. 1990; Matilla et al. 2000). Besides, SAM is the source of the propylamine 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 (Kaur-Sawhney et al. 2003; Kuznetsov and Shevyakova 2007; Pang et al. 2007). SAM is also the precursor for the metal ion chelating compounds, nicotinamide and phytosiderophores, as well as for the B vitamin, biotin (Ravanel et al. 1998a; Droux 2004; Hesse et al. 2004; Roje 2006) (Fig. 1). Methionine itself serves as a donor for various secondary metabolites such as glucosinolates, which are involved in pathogen and insect defense, and are produced predominantly in plants belonging to the Brassicaceae family (Gigolashvili et al. 2007; Hirai et al. 2007). Finally, methionine leads to the synthesis of S-methylmethionine (SMM), which is considered to be the mobile and storage form of methionine (Mudd and Datko 1990), the regulator of SAM level in cells (Ranocha et al. 2001; Kocsis et al. 2003), and a precursor to other secondary metabolites (Hanson et al. 1994).

METHIONINE METABOLISM IN PLANTS
Based on the above data, the optimization and elevation of methionine levels in crop plants for animal feed and human food must also consider the other essential functions of methionine in plant metabolism. Hence, in order to further enhance methionine levels in different plant tissues, the methionine metabolism must be studied intensively. The points of methionine regulation along its biosynthesis and its catabolism must be elucidated in order to tailor its optimal level in different tissues without causing an abnormal phenotype that may have negative effects on plants.

Two main strategies have been used to increase methionine content in plants using recombinant DNA technologies through the manipulation of the methionine biosynthetic pathway and by the creation of additional protein ‘sinks’ for methionine storage. Both molecular approaches (as described below) have provided new insights into the control of methionine levels in plants, and in some cases, have yielded significantly higher levels and thus improved nutritional value in plants.

Most of the preliminary approaches to elevate the methionine level in plants are based on the knowledge that methionine in plants has a relatively short half-life due to its rapid metabolism (Giovaneli et al. 1985; Miyazaki and Yang 1987). Giovaneli and associates measured the incorporation of sulfate, methyl and carbon skeleton into methionine and its products in Lemma, an aquatic plant, using tracer elements. They observed that methionine is converted to SAM about four times faster than it is incorporated into proteins (Giovaneli et al. 1985). In mature Arabidopsis rosette leaves, however, Ranocha et al. (2001), who used radioactive methionine and in silico modeling, suggest that about half of the soluble methionine converts to SAM and SMM, and half to protein synthesis. These studies suggest that methionine levels can be elevated by reducing the flux towards SAM, leaving more methionine molecules availa-
Attempts to increase methionine content in plants. Rachel Amir

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Attempts to improve the methionine level by reducing the expression level of SAM synthase

Two studies analyzing Arabidopsis mutants (mto3), in which one of its isozymes for SAM synthase exhibits a reduced expression level, have shown that methionine content increased significantly to over 200-fold compared to wild-type plants (Goto et al. 2002; Shen et al. 2002). However, this reduction was accompanied by a low content of lignin, one of the major metabolic sinks for SAM (Shen et al. 2002). A more severe phenotype appeared in SAM synthase-silenced Arabidopsis plants. In these plants, the methionine level increased 250-fold compared to wild-type plants, but severe phenotype abnormalities were observed (Kim et al. 2002). A severe abnormal phenotype also appeared in tobacco plants where the SAM synthase level was significantly reduced (Boerjan et al. 1994). These results suggest that a reduction in the expression level of SAM synthase could significantly increase the methionine level, but due to the importance of SAM for plant metabolism, such a reduction could lead to severe abnormal morphological phenotypes. Therefore, it is not expected that this approach for increasing soluble methionine will be used in the future.

Attempts to improve the methionine level by expressing methionine-rich storage proteins in transgenic plants

The second option for competing with the rapid flux of methionine towards SAM and its metabolites was to express methionine-rich storage proteins in transgenic plants, creating a new ‘sink’ for the soluble methionine. This approach was studied extensively both in seeds and vegetative tissues as described below (reviewed by Tabe and Higgins 1998; Galili et al. 2005; Amir and Tabe 2006).

In most cases, genes for methionine-rich 2S albumin storage proteins were used, particularly Brazil nut 2S albumin, which contains 18% methionine, and a 2S albumin from sunflower, which contains 16% methionine residues. The Brazil nut 2S albumin was expressed in seeds of a number of plant species, including tobacco, canola, narbon bean and soybean. In some cases, as expected, a significant enhancement in total seed methionine was observed compared to wild-type seeds (Altenbach et al. 1989, 1990, 1992; Saalbach et al. 1995; Muntz 1997; Tabe and Higgins 1998). In the cases of soybean and narbon bean, this improvement brought the proportion of methionine in seed protein close to that required by animals for optimal growth (Altenbach et al. 1989, 1992; Muntz et al. 1997; Tabe and Higgins 1998). The methionine content of seed protein was also doubled in the feed legume grain, narrow leaf lupin, through the expression of the sunflower 2S albumin. In this case, however, the improved methionine level was still short of the animal growth requirement (Molvig et al. 1997;
Tabe and Higgins 1998). Unfortunately, the 2S albumins from the Brazil nut and the sunflower was found to be allergenic to some people (Bartolome et al. 1997; Kelly and Hefle 2000), reducing the usefulness of these proteins in crop plants, at least those required for human nutrition. The allergenic problem forced the researchers to seek new methionine-rich protein candidates. This led to the identification of protein that is rich in all essential amino acids including 20% methionine and 13% cysteine (Wandelt et al. 1995, 2004). Co-expression of these two proteins together also significantly further increased the level and stability of the δ-zein, implying that interactions between different zeins may be important for their accumulation (Baga et al. 1997; Kim et al. 2002; Baga et al. 2004).

Attempts to improve the methionine level by co-expressing methionine-rich storage proteins with enzymes that lead to high soluble methionine levels

The studies described above, although they resulted in some cases in a significant increase in total methionine in protein (Altenbach et al. 1992; Baga et al. 1997; Molvig et al. 1997), show in other plant species that this increase in methionine was at the expense of other sulfur compounds or endogenous methionine-rich proteins (Muntz 1997; Tabe and Higgins 1998; Hagan et al. 2003; Chiaiese et al. 2004). To further test whether the level of soluble methionine limits the production of sulfur-rich proteins in vegetative tissues, the transgenic alfalfa plants constitutively expressing the 15 kD zein were crossed with those exhibiting significant higher methionine content due to the constitutive expression of the Arabidopsis cystathionine γ-synthase (CGS) and bacterial aspartate kinase (AK) lines (Avrahem et al. 2005b). Compared to plants expressing only the 15 kD zein, those co-expressing both transgenes showed significantly enhanced levels of the maize 15 kD zein concurrently with a reduction in the level of soluble methionine, implying that in the crossed plants more soluble methionine was incorporated into the 15 kD zein (Baga et al. 2005; Golan et al. 2005). These studies also show that the soluble methionine content may limit the accumulation of methionine-rich storage proteins. The elevation of the 15 kD zein in alfalfa plants is of particular nutritional importance to ruminant animals, since this protein is resistant to rumen proteolysis (Baga et al. 2004). Similar phenomena also occur in tobacco expressing these two genes, but they are considerably less pronounced. The results showed that the accumulation of the β-zein is regulated in a species-specific manner and that soluble methionine plays a major role in the accumulation of the β-zein in some plant species but less so in others (Baga et al. 2005; Golan et al. 2005).

To further study whether the soluble methionine level also limits the synthesis of sulfur-rich-storage proteins in seeds, Demidov et al. (2003) produced seeds of Vicia narbonensis that expressed both the Brazil nut 2S albumin protein and bacterial aspartate kinase (AK), both controls under a seed-specific promoter. Expression of the feedback-inhibitory AK in transgenic tobacco seeds resulted in three-fold increases in soluble methionine in mature homozygous seeds (Karchi et al. 1993). The AK lines had 10 to 12 % and the Brazil nut 2S albumin lines 80% increased methionine in mature seeds. The double transformants exhibited additive effects on seed methionine content, showing that in mature seeds the protein-bound seeds reached levels 2.4 times higher than the wild-type seeds. These results suggest that in vivo the AK in tobacco seeds resulted in three-fold increases in soluble methionine in mature homozygous seeds. The findings described above indicate that cysteine and methionine availability limit the enrichment of seed proteins with sulfur amino acids in plants, and hence it has been 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; Tabe and Droux 2002). Several approaches have been tested and are described below.

APPROACHES TO IMPROVE METHIONINE CONTENT BY INCREASING ITS SOLUBLE LEVEL

The findings described above indicate that cysteine and methionine availability limit the enrichment of seed proteins with sulfur amino acids in plants, and hence it has been 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; Tabe and Droux 2002). Several approaches have been tested and are described below.
Attempts to increase the soluble methionine content through overexpression of cystathionine γ-synthase (CGS)

Several molecular and biochemical lines of evidence have suggested that the first unique enzyme of the methionine biosynthesis pathway, cystathionine γ-synthase (CGS), which combines the carbon/amino skeleton derived from the threonine branch of the aspartate family with the sulfur moiety derived from cysteine (Giovanelli et al. 1980; Ravanel et al. 1995; Kim et al. 1996), plays a major role in determining the methionine level in plants. Reducing the expression level of this enzyme significantly lowered the methionine content in plants (Ravanel et al. 1998a), resulting in severe growth retardation, while overexpression of the Arabidopsis gene encoding for this enzyme led to a significantly higher methionine level in tobacco (Hacham et al. 2002; Hacham et al. 2006). Arabidopsis (Kim et al. 2002), potato (Di et al. 2003) and alfalfa plants (Avraham et al. 2005b).

Contrary to 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. 1998a, 1998b). However, it was found that the methionine downstream product, SAM, negatively regulates the transcript level of Arabidopsis CGS (AtCGS), keeping the methionine level under tight control (Chiba et al. 1999, 2003; Onouchi et al. 2004, 2005). This regulation occurs in the N-terminal region of AtCGS comprised of about 100 amino acids (without its plastid transit peptide), which does not exist in bacterial enzymes and is not essential for the catalytic activity of AtCGS. Mutations in the sub-domain of this N-terminal region led to a 40-fold higher accumulation of methionine than for wild-type Arabidopsis plants. Hence this domain was called MTO1 for methionine over-accumulation (Chiba et al. 1999; Inba et al. 1994). A detailed analysis of this MTO1 domain reveals that SAM induces a temporal arrest in the translation elongation process during AtCGS mRNA translation of the MTO1 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 (Onouchi et al. 2005; Haraguchi et al. 2008). These studies demonstrated that the CGS transcript level was controlled by SAM content, and that the methionine synthesis in Arabidopsis plant cells was controlled by the amount of CGS transcript. The results of these studies also suggested that overexpression of the mutated form of AtCGS (without the 90 nt domain) in transgenic plants can lead to significantly higher levels of AtCGS and thus to a higher methionine content in these plants. Surprisingly, although this approach to elevate methionine content seems very promising, it has not yet been tested.

Overexpressing the truncated form of AtCGS that lacks the N-terminal region and thus the MTO1 domain in transgenic plants leads to a severe phenotype compared to plants overexpressing the full-length AtCGS (Hacham et al. 2002). Plants expressing this truncated form also have a strong bad smell comprises of dimethylsulfide and carbon disulfide, two catabolic products of methionine, and they emitted a high level of ethylene, a SAM metabolite (Hacham et al. 2002). Therefore, the removal of the N-terminal region is not an appropriate approach for increasing methionine levels in these plants. Indeed, 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 these plants does not play a major role in controlling methionine level (Kreft et al. 2003). This observation also suggests that potato is missing some elements required for post-transcriptional of CGS regulation to occur, and that regulation through the MTO1 region is insufficient. Since potato CGS is insensitive to methionine application using the potato form of CGS it also should be considered for increasing methionine content in transgenic plants. Taken together from the results described above, two forms of AtCGS can be used to increase methionine content in plants: AtCGS that contains the MTO1 specific mutations (Chiba et al. 1999), and the deleted form of AtCGS that lack the 90-nt domain (Hacham et al. 2006). The use of potato CGS could also be considered.

Unlike CGS, which plays a major regulatory role in methionine synthesis, at least in Arabidopsis, no direct evidence shows that the levels of the second and third enzymes of the methionine biosynthesis pathway, cystathionine β-lyase and methionine synthase, play a regulatory role in determining methionine content in plants. Indeed overexpression of these two enzymes did not lead to an increase in flux towards methionine synthesis in tomatoes (Maimann et al. 2001; Nikiforova et al. 2002; Hesse and Hofgen 2003).

Attempts to increase the soluble methionine content through a reduction in threonine synthase (TS)

Several lines of evidence have shown that methionine biosynthesis is also regulated by the competition between CGS and TS, the last enzyme in the threonine pathway, for their common substrate O-phosphohomoserine (OPH) (Fig. 1) (reviewed by Amir et al. 2002; Hesse et al. 2004). In vitro activity measurements indicate that TS in plants has 250- to 500-fold higher affinity for OPH compared to AtCGS, causing reduced OPH availability for methionine synthesis (Curien et al. 1998; Ravanel et al. 1998a). However, a modeling analysis recently 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 seven-fold higher than the AtCGS level, causing the flux towards threonine synthesis to be four-fold higher than the flux towards methionine (Curien et al. 2003).

According to these analyses, it was expected that a lower TS level would lead to higher OPH available to CGS, and as a result, the level of methionine will be increased. Indeed, analyses of transgenic and mutant plants having lower TS protein levels or lower TS activity have shown that the level of methionine significantly increased. For example, when the TS activity was reduced due to mutation in Arabidopsis plants, the level of methionine increase 22-fold in rosette leaves of mutant compared to wild-type plants (Bartlem et al. 2000). Similarly, a reduction in TS levels using an antisense approach in potato and Arabidop-
six plants caused a significant increase in methionine level by 239- and 47-fold, respectively (Zeh et al. 2001; Avraham et al. 2005a). Although the level of methionine significantly increased in these plants, from a biotechnological point of view, this approach is inappropriate since the level of threonine, which is important in limiting essential amino acids, was reduced significantly in these plants.

To overcome the point of regulation between TS and CGS (Fig. 1) and acetyl-CoA to form O-acetylhomoserine, a metabolite not produced in plants. O-acetylhomoserine can be used by the plant’s CGS, as shown by in vitro (Ravanel et al. 1998b), and in vivo (Hacham et al. 2003) studies. Thus, expression of this enzyme in plants can potentially bypass the TS/CGS competition, channeling more carbon/amino skeleton from homoserine towards methionine synthesis. However, the result indicates that the bacterial and yeast enzymes are heat labile and tend to change their intracellular conformation.

As a result, O-acetylhomoserine was not detected in transgenic plants, and the approach was not considered for use (Ganrasaki et al. 2005). The importance of the OPH level in the methionine and threonine biosynthesis pathway and their accumulation was recently shown in wild-type and transgenic Arabidopsis plants overexpressing homoserine kinase fed with homoserine. The levels of both amino acids increased significantly in these plants (Lee et al. 2005). 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. This probably occurs due to the close regulation of the first unique enzyme of the aspartate family, aspartate kinase (AK), and homoserine dehydrogenase activities that limit OPH production. All in all, the results described above indicate that methionine content is tightly regulated by the flux of the carbon/amino skeleton towards its synthesis, and that a strategy to increase methionine content might involve the co-expression of CGS along with the feedback-insensitive mutant form of AK.

**Attempts to increase the soluble methionine content through the expression of the insensitive form of AK together with AtCGS**

Several studies indicate that the carbon/amino skeleton flux towards the threonine branch of the aspartate amino acid family is controlled by the activity of AK (reviewed by Azevedo et al. 2006). Mutants possessing feedback-insensitive AK (Frankard et al. 1991) and those overexpressing the E. coli feedback-insensitive AK enzyme in transgenic tobacco (Shaul and Galli 1992a), Arabidopsis (Ben Tzvi et al. 1996) and alfalfa plants (Galli et al. 2000) resulted in a significant overproduction of free threonine, while methionine content, whose biosynthesis pathway diverges from this branch, does not differ significantly. A slight but significant elevation in methionine content was found when this bacterial enzyme was expressed in a seed-specific manner (Karchi et al. 1993). In order to further study the regulatory role of the carbon/amino flux towards methionine synthesis, tobacco plants overexpressing the bacterial AK (Shaul et al. 1992) were crossed with those overexpressing the full-length AtCGS (Hacham et al. 2002). Plants co-expressing these two genes 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 the full-length AtCGS alone (Hacham et al. 2008). This finding contradicted that suggested by Lee et al. (2005) whereby the overexpression of CGS coupled with the feedback-insensitive form of AK can contribute to methionine production, compared to plants expressing only the full-length CGS. However, the result could be explained by the feed-back-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 were deleted (Hacham et al. 2002). These two plants were methionine/SAM insensitive. Indeed, significantly higher methionine contents accumulated in the newly-produced plants compared to plants expressing these forms of AtCGS alone (about 4.5-fold higher), while the level compared to wild-type plants increased to about 110- to 190-fold higher. The threonine levels doubled in these plants compared to wild-type plants (Hacham et al. 2008). In the latter plants, the level of most amino acid contents increased. This implies that the content of methionine or one of its associated metabolites beyond a certain threshold may serve as a signal and affect other biosynthesis pathways. Among the amino acids whose levels increased were aspartate, the donor of the carbon/amino skeleton for methionine and threonine synthesis, and lysine and isoleucine, which belong to the aspartate family. Taken together, these results suggest that the carbon/amino skeleton limits methionine synthesis, but this can hardly be seen under normal growth conditions since the regulatory role of methionine/SAM on the expression level of AtCGS has a relatively strong effect. Therefore, identification of regulatory elements in CGS and their removal are important for producing plants with a higher level of methionine. 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 limits the nutritional value of cereals), together in the same plant tissue, without leading to the formation of abnormal phenotypes.

**Attempts to increase the soluble methionine content through the expression of enzyme from the cysteine biosynthesis pathway together with AtCGS**

CGS in higher plants combines the carbon/amino skeleton with the sulfur group donated from cysteine. While, as described above, the carbon/amino skeleton level significantly limits methionine synthesis, it is still unclear if the sulfur level, and more specifically, cysteine content, limits methionine synthesis in plants. Plants having higher levels of cysteine due to manipulations of the cysteine biosynthesis pathway, such those overexpressing genes encoding to its two last biosynthesis enzymes, serine acetyl transferase or O-acetylserine (thiol) lyase, lead to higher cysteine and glutathione levels (Saito et al. 1994; Blaszczyk et al. 1999; Harms et al. 2000; Noji et al. 2001; Nikiforova et al. 2002; Wirtz and Hell 2003; Matityahu et al. 2005; Stiller et al. 2007). However, the level of methionine in these transgenic plants (when measured) was not significantly altered (Matityahu et al. 2005). This suggests that under natural conditions, cysteine channeled towards glutathione synthesis, whereas the incorporation of cysteine into methionine synthesis is tightly controlled, most probably by the level and activity of CGS. The observation that transgenic plants exhibiting a higher soluble level of methionine emitted higher levels of catabolic products of methionine that contain sulfur, such as methanethiol, dimethylsulfide and carbon disulfide (Boerjan et al. 1994; Hacham et al. 2002), also suggested that the sulfur compounds and cysteine content do not limit methionine synthesis in plants.

To further test this assumption, plants overexpressing the yeast O-acetyl(thiol)lyase (yOASTL) (Matityahu et al. 2005) were recently crossed with those overexpressing the full-length AtCGS (Hacham et al. 2006). Plants overexpressing the yeast gene both in the cytosol and the chloroplasts exhibit high levels of cysteine and glutathione in their leaves (Matityahu et al. 2005). Plants overexpressing the
chloroplast form of yOASTL and AtCGS (which is naturally active in the chloroplasts) showed slightly but significantly higher amounts of methionine and the methionine metabolite, SMM, compared to plants overexpressing the AtCGS alone. However, the levels of methionine and SMM are not altered in plants overexpressing the AtCGS and the yeast protein targeted to the cytosol compared to the levels found in their corresponding parents (R. Amir, unpublished results). Expression of both sets of enzymes in tobacco plants overexpressing AtCGS that exhibit significantly increased compared to the level found in wild-type plants. Taken together, the results suggested that in the chloroplasts, where the first enzyme for methionine is localized, higher levels of cysteine could contribute to methionine synthesis when a high expression level of CGS exists and the levels of methionine and cysteine can accumulate in both plant tissues.

Interestingly, it was found that alfalfa overexpressed the AtCGS, which exhibits significantly higher levels of methionine and SMM as well as methionine that was incorporated into the water-soluble protein fraction (32-fold, 19-fold and cysteine, and protein-bound cysteine increased up to 2.6-fold, 5.5-fold and 2.3-fold, respectively, relative to wild-type plants (Avraham et al. 2005b). An examination of the sulfate level in leaves of these plants demonstrated that they contain significantly higher levels of sulfate compared to wild-type plants (R. Amir, unpublished results). This suggests that an elevation in the level of AtCGS enhanced the sulfate uptake and sulfur assimilation to sulfur-amino acids, which eventually led to higher levels of these amino acids in the leaves’ proteins. Further studies will be required if this phenomenon also occurs in other legumes and crop plants and whether sulfur fertilizing would enhance the levels of these two amino acids later on.

Attempts to increase the soluble methionine content together with lysine, an important and nutrition-limiting essential amino acid

The content of lysine, an essential amino acid, limits the nutritional value of many crop plants, especially those of cereal grains such as wheat, corn and rice, which represent the major source of human food and animal feed worldwide (Galili et al. 2005; Shaul et al. 2006b). The lysine biosynthesis pathway exists in other branches of the aspartate family of amino acids leading to threonine, methionine and isoleucine synthesis (Fig. 1). The regulation of the carbon/amino skeleton flux within these two branches is quite complex, and metabolites from one branch affect flux towards the other (Galili 1995; reviewed by Azevedo et al. 2006). In an attempt to reveal the crosstalk between metabolites and genes belonging to the aspartate family, and to explore the regulation of methionine metabolism, the transgenic tobacco plants overexpressing AtCGS that exhibit higher levels of methionine were crossed with those overexpressing the feedback-insensitive bacterial enzyme dihydrodipicolinate synthase (bDHPS) that contains a significantly higher level of lysine (Shaul and Galili 1992b). Since the lysine pathway diverges earlier than the methionine pathway within the aspartate family, it was expected that enhanced flux of the carbon/amino skeleton towards lysine would reduce the level of methionine in these plants. However, when expressing both foreign genes, it was found unexpectedly that the methionine levels were significantly elevated compared to those expressing AtCGS alone, while the level of lysine remained the same as those overexpressing bDHPS alone (Hacham et al. 2007). The increased levels of methionine and SMM correlated with the elevation in mRNA and protein levels of AtCGS and with the reduced mRNA level in genes encoding for SAM synthase, which converts methionine to SAM. Taking these results into account, the following scheme was proposed for the crosstalk between lysine and methionine biosynthesis pathways (Fig. 1, dotted line). A high level of lysine brings about a reduction in the amount of enzyme SAM synthase due to a reduction in the amount of transcripts encoding this enzyme. This leads to a reduction in the amount of SAM, which negatively regulates the amount of AtCGS transcript (Chiba et al. 2003). As a result, the expression level of AtCGS is increased and consequently the level of methionine (Hacham et al. 2007). The increased methionine level can also be enhanced by a reduction in flux towards SAM and its metabolites (Giovanelli et al. 1985). Taken together, this mechanism ensures a fine balance between the amounts of lysine and methionine through an indirect biochemical crosstalk mechanism involving the regulation of the expression of SAM synthase.

The finding that high lysine content was associated with higher methionine levels in the same plant tissue was also observed in other studies. Three sets of Arabidopsis transgenic plants exhibiting significantly higher levels of lysine in their seeds resulting from seed-specific expression of bDHPS and RNAi of lysine-ketogluarate reductase/saccharopine dehydrogenase, the catabolic enzyme of lysine (Zhu and Galili 2003, 2004), have shown, in addition to a significant 80-fold increase in lysine content, that the methionine level increased significantly, up to 51-fold compared to wild-type Arabidopsis. The relation between high lysine and methionine levels was also found in transgenic barley plants that constitutively express the bDHPS. These plants exhibited a 14-fold increase in free lysine and a 8-fold increase in free methionine (Brinch-Pedersen et al. 1996). These results show that, as seeds, as well as in vegetative tissues, a higher level of lysine enhances the production of methionine or reduces its catabolism.

From a biotechnological point of view, the results described here show that significantly higher methionine contents can appear with significantly higher lysine levels. The latter two amino acids are essential amino acids that limit the nutritional value of cereal grains where a low level of methionine is also found (Galili et al. 2005; Shaul et al. 2006). Notably, in these plants the levels of threonine and isoleucine belonging to the aspartate family are not reduced and even slightly increase. Thus, these studies present new ways of manipulating the levels of these amino acids in plants in order to increase their nutritional value. However, this manipulation in crop plants should be considered carefully, since in these plants the level of SAM is significantly reduced, which might affect the level of essential methionine/SAM metabolites. Reduction in SAM content can lead, for example, to lower lignin content (as described above for the mto3 mutants) (Shen et al. 2002).

The soluble methionine content might also be increased by reducing the expression level of methionine γ-lyase, the methionine catabolic enzyme

It was recently found that Arabidopsis has a similar sequence to the bacterial gene encoding the methionine γ-lyase gene. Two groups of researchers cloned the cDNA of this gene (Rebeille et al. 2006; Geyer et al. 2007) and found that this cytosolic enzyme is abundant in all plant organs except in the seeds, and it catalyzes the conversion of methionine into methanethiol, α-ketobutyrate and ammonia. Western blot studies have indicated that this gene is expressed under standard growth conditions and was strongly induced by recycling of the cells accumulated methionine (Rebeille et al. 2006). The enzyme has a relatively high Km level for methionine (~10 mM), indicating that this pathway operates preferentially when methionine has accumulated above a certain value in the cytoplasm. Knocking out the methionine γ-lyase gene in Arabidopsis significantly increased leaf methionine content (9-fold) and SMM content under sulfate starvation, but did not affect methionine level under normal growth conditions (Geyer et al. 2007). This finding suggests that this catabolic pathway plays a role during sulfate starvation.
starvation, but since the level of methionine is not altered under this stress (Nikiforova et al. 2005; Nikiforova et al. 2006), the situation is not clear and further studies are required. The question still remains regarding the role of this enzyme when a high level of methionine increases in plants such as in plants overexpressing the AtCGS (Avraham et al. 2005a; Hacham et al. 2006), since in some cases the level of methanethiol and other catabolic products of methionine (e.g., (S)-methylisovalerate, dimethylpropane) significantly increased (Boerjan et al. 1994; Hacham et al. 2002). Further studies are required in order to reveal this point of regulation.

Higher levels of methionine in Arabidopsis seeds were found in HMT2 mutant

Analyses of Arabidopsis transgenic plants overexpressing AtCGS and Arabidopsis mutants, mto-1 and mto-2, have shown that a relatively high level of methionine can be found in young rosette leaves. However, this accumulation is strongly dependent on development stage and organs (Inba et al. 1994; Bartlem et al. 2000; Kim et al. 2002). As the mutant plants began to flower, the levels of methionine and SMM declined gradually in the leaves while a high level of methionine, homocysteine and cysteine was found in the reproductive tissues (Inba et al. 1994; Bartlem et al. 2000; Kim et al. 2002). These findings suggest that the soluble methionine accumulation in leaves during the vegetative growth period was translocated to the sink organs at the onset of reproductive growth, and that methionine is most probably not synthesized in situ in these organs. Experiments using radioactive methionine have indeed shown that methionine exports from leaves in the form of SMM, which is the major sulfur-metabolite in the phloem of different plants (Bourgis et al. 1999). Moreover, the results suggested that SMM is the major donor for methionine required for the synthesis of proteins in wheat seeds (Bourgis et al. 1999).

SMM is formed from methionine via the activity of methionine methyltransferase (MMT), which uses SAM as a methyl group donor to form SMM from methionine. SMM then converts to two methionine molecules by using homocysteine and SMM, reactions that are catalyzed by homocysteine S-methyltransferase (HMT) (Mudd and Datko 1990; Ranocha et al. 2001) (Fig. 1). These two enzymes comprised the SMM cycle in plants. Each turn of the cycle hydrolyzes ATP, and thus this cycle, is considered to be futile. Hence, the assumption proposed by Bourgis et al. (1999) that SMM is the major donor for methionine in wheat seeds demands a complete separation in space and time on methionine demand and SMM transport in seeds, which is the major sulfur-metabolite in the phloem of different plants (Bourgis et al. 1999). Moreover, the results suggested that SMM is the major donor for methionine required for the synthesis of proteins in wheat seeds (Bourgis et al. 1999).

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Support for the hypothesis that SMM transports towards the developing seeds from vegetative tissues recently came from the finding that in the hmt2 mutant, a significantly high level of methionine was found in seeds. As a result of the hmt2 mutation, a significant accumulation of SMM was observed in the upper flower stalk (cauline leaves, stems, flowers, silique hulls and seeds) of the mutant compared to wild-type plants (Lee et al. 2008). The results obtained using [13C]SMM and [13C]methionine suggests that due to increased accumulation at the top of the hmt2 flower stalk, SMM transport towards seeds is greatly increased, which then converts to methionine by seed HMT isozymes (other than HMT2), whose level is not reduced in the mutant compared to wild-type plants (Lee et al. 2008). This study suggests that manipulation of the SMM cycle may provide a new approach for improving the methionine level in seeds. However, further studies are required to reveal the importance of SMM cycle since it was shown that the lack of SMM cycle in the mmt mutant of Arabidopsis does not alter the levels of methionine, thiol and seed sulfur content compared to wild-type seeds (Koessis et al. 2003). This suggests that other sulfur sources such as glutathione or sulfate can replace SMM as a long-distance transporter.

In addition to the role of SMM cycle in the accumulation of methionine in seeds, methionine is most probably also synthesized in seeds through the CGS activity since the enhanced flux of the carbon/amino skeleton towards its biosynthesis pathway, caused by the expression of bacterial AK under the seed-specific promoter, possesses small but significant increases of methionine content compared to wild-type seeds (Karchi et al. 1993; Demidov et al. 2003).

**FUTURE PROSPECTS**

Although major progress has been made in recent years to increase methionine content in vegetative and seed tissues of plants, understanding the factors that regulate the methionine level in these tissues is not completely known and further study is required. It is expected that in the future, a combination between methionine-rich storage proteins and genes encoding for enzyme/s that lead to a high level of soluble methionine will be required in elevating the total methionine content in plants and adapting them to human and animal nutrition. While efforts to express methionine-rich storage proteins should be concentrated mainly in searching for new catalytic forms that do not catabolize methionine and to study their effects on methionine accumulation mainly in legume crop plants, ways of increasing the soluble level require more extensive studies and better knowledge about the methionine metabolism. At this point, the use of AtCGS, which its regulatory domains are omitted (like that lack the 90 bp domain), or the specific mutations in the MTO1 region, are the most appropriate candidates for this mission. The combination of the unregulated forms of AtCGS with feedback insensitive form of AK, can lead to further methionine enhancement, coupled with a high threonine level. Combination of the unregulated forms of AtCGS with methionine γ-lyase might also lead to high methionine content and should be consider mainly in vegetative tissues. These approaches are important for controlling the methionine level in plant tissues hoping that they lead to minimal abnormal phenotypes of transgenic crop plants.

The role of methionine and SMM transport from vegetative tissues towards the seeds should also be studied in the future, since it is not clear whether methionine can be synthesized directly in seeds. In addition to SMM, the roles of sulfate transport and sulfate transporters, their sub-cellular localization and their significance in balancing sulfur flux to sulfur demand of the plant's organs should be elucidated. The role of the sulfur-fertilizing regime during plant development should be studied in order to support optimal methionine synthesis during the various developmental stages in the different transgenic lines, as well as in the wild-type.

Since methionine is comprised of three different moieties (carbon/amino skeleton, sulfur group, and methyl group), three different biochemical pathways contribute to its synthesis suggesting that the coordination between these pathways might be complicated and require regulatory mechanisms. Moreover, methionine is required for the production of various essential metabolites in plants, in addition to its role in protein synthesis, and therefore its metabolism is quite complicated. Two recycle pathways (in addition to the SMM cycle) can regenerate the moieties that comprise methionine following the formation of SAM metabolites (Fig. 1): (i) the methyl cycle, in which the methyl group of methionine/SAM is donated to methyl transfer reactions, leaving S-adenosylhomocysteine, which recycles to methionine through homocysteine and the activity of the cysteoytic form of methionine synthase that combines the methyl group to homocysteine to regenerate methionine (Roje et al. 2006); and (ii) the Yang cycle (also termed the MTA cycle), where ethylene, biotin and polyamines are synthesized, and the methylthio moieties are recycled to methionine via methyliobutyrate (Fig. 1) (Yang and Hoffman 1990). Although the importance of these pathways is accepted, little is known about their regulation and contribution to the...
methionine pool, and how their rate is changed as a result of various conditions, such as sulfur starvation, different levels of sulfur in fertilization, biotic and abiotic stresses, and their role during plant development. Little is also known about the role of protein degradation and how it supports the methionine steady-state level in cells under these conditions. Hence, in the future it is expected that more data about the methionine metabolism will be found that could lead to additional points of regulation for increasing the methionine level.

Genomics approaches, such as gene expression profiling in microarrays, proteomics, metabolic profiling and flux analysis measurements, will greatly contribute to our knowledge in the future. Such approaches are already being used extensively regarding the metabolism of sugars, lipids and secondary metabolites, and it is expected that they will strongly penetrate into the metabolism of amino acids and sulfur metabolites. In addition to furthering our knowledge about plant metabolism, this knowledge will help in manipulating the methionine metabolism and in crop plants having higher levels of methionine, thus improving nutritional quality.

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