Lysine enhances methionine content by modulating the expression of S-adenosylmethionine synthase

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Summary

Lysine and methionine are two essential amino acids whose levels affect the nutritional quality of cereals and legume plants. Both amino acids are synthesized through the aspartate family biosynthesis pathway. Within this family, lysine and methionine are produced by two different branches, the lysine branch and the threonine–methionine branch, which compete for the same carbon/amino substrate. To elucidate the relationship between these biosynthetic branches, we crossed two lines of transgenic tobacco plants: one that overexpresses the feedback-insensitive bacterial enzyme dihydrodipicolinate synthase (DHPS) and contains a significantly higher level of lysine, and a second that overexpresses Arabidopsis cystathionine γ-synthase (AtCGS), the first unique enzyme of methionine biosynthesis. Significantly higher levels of methionine and S-methylmethionine (SMM), accumulated in the newly produced plants compared with plants overexpressing AtCGS alone, while the level of lysine remained the same as in those overexpressing DHPS alone. The increased levels of methionine and SMM were correlated with increases in the mRNA and protein levels of AtCGS and a reduced mRNA level for the genes encoding S-adenosylmethionine (SAM) synthase, which converts methionine to SAM. Reduction in SAMS expression level leads most probably to the reduction of SAM found in plants that feed with lysine. As SAM is a negative regulator of CGS, this reduction leads to higher expression of CGS and consequently to an increased level of methionine. Elucidating the relationship between lysine and methionine synthesis may lead to new ways of producing transgenic crop plants containing increased methionine and lysine levels, thus improving their nutritional quality.

Keywords: methionine metabolism, aspartate family, cystathionine γ-synthase, dihydrodipicolinate synthase, S-adenosylmethionine (SAM), SAM synthase.

Introduction

Lysine, methionine and threonine are of great nutritional importance in animal feeds and human foods because of the limited amounts of these essential amino acids in seeds and vegetative tissues of many crop plants (recently reviewed by Azevedo et al., 2006; Galili et al., 2005; Hesse and Hoefgen, 2003; Hesse et al., 2004). These three essential amino acids are synthesized in plants from aspartate via two branches of the aspartate family biosynthetic pathway: the lysine branch and the threonine–methionine–isoleucine branch (Figure 1).

The aspartate family biosynthesis pathway in plants is regulated by several feedback inhibition loops. Molecular and biochemical studies have shown that the isoforms of aspartate kinase (AK), the enzyme catalyzing the first step in this pathway, are feedback-inhibited by several products along the biosynthesis pathways. Threonine negatively regulates the activity of AK–homoserine dehydrogenase (Curien et al., 2005), while lysine negatively regulates the activity of the monofunctional AK isoforms (Frankard et al., 1997; Tang et al., 1997). Moreover, in vitro studies have demonstrated that lysine feedback inhibition is synergistically enhanced by S-adenosylmethionine (SAM), a methionine metabolite, but SAM by itself does not affect the activity of AK (Rognes et al., 1980). In addition, lysine also inhibits the activity of the first enzyme in its own pathway,
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Methionine synthesis is regulated by the level of its first specific biosynthesis enzyme, cystathionine γ-synthase (CGS), and by the level of threonine synthase (TS), the last enzyme in the threonine biosynthesis pathway (Amir et al., 2002; Avraham and Amir, 2005; Chiba et al., 1999; Droux et al., 2000; Galili et al., 2005; Hesse and Hoefgen, 2003; Hesse et al., 2004; Ravanel et al., 1998; Zeh et al., 2001). These two enzymes compete for their common substrate, O-phosphohomoserine (OPH; Figure 1). In vitro biochemical studies revealed that the affinity of TS for OPH is stimulated by SAM (Curien et al., 1998), suggesting that methionine regulates its own synthesis through SAM. When the level of methionine, and hence SAM, is high, TS activity is increased, causing reduced OPH availability for methionine synthesis (Curien et al., 1996, 1998). In addition, in Arabidopsis, SAM also negatively regulates the transcript level of CGS (Chiba et al., 1999, 2003; Kreft et al., 2003; Onouchi et al., 2005). Therefore SAM plays a major role in the regulation of methionine and threonine synthesis and affects methionine accumulation in plants. In addition to these effects on the regulation of methionine synthesis, the level of methionine is also regulated by the rate of its catabolism to SAM and to SAM metabolites (Giovanelli et al., 1985). A reduction in the amount or activity of SAM synthase (SAMS), the enzyme that converts methionine to SAM, resulted in a significantly higher level of methionine in these plants (Boerjan et al., 1994; Goto et al., 2002; Shen et al., 2002).

In an attempt to reveal the cross-talk between metabolites and genes that belong to the aspartate family, and to explore the regulation of methionine metabolism, we address here the following questions: (i) considering that the lysine and methionine biosynthesis pathways are located on different branches of the aspartate family biosynthesis pathway, how does the methionine level change when the flux towards lysine synthesis is significantly enhanced? and (ii) can lysine and methionine accumulate to a significantly high level in the same plant tissue? The latter is a particularly important issue from a biotechnological standpoint. To this end, we crossed tobacco plants overexpressing bacterial DHPS and those overexpressing Arabidopsis CGS (AtCGS). The results disclosed a new regulatory point at which lysine affects methionine content by modulating the expression level of the genes encoding the enzyme SAMS. In addition, we show that both methionine and lysine can significantly over-accumulate in the same plant tissue.

**Results**

*Methionine level is enhanced in tobacco plants overexpressing both Arabidopsis CGS (AtCGS) and bacterial DHPS compared with plants overexpressing AtCGS only*

An increase in lysine content has been reported for plants overexpressing the *Escherichia coli* DHPS enzyme (Karchi...
et al., 1994; Perl et al., 1992; Shaul and Galili, 1992a). In these plants, lysine accumulation reflected the amount of the enzyme as well as its feedback-inhibition insensitivity, unlike its plant counterpart (Galili, 1995). With regard to methionine, we have previously obtained a significant accumulation of this amino acid, as well as its metabolite S-methylmethionine (SMM), in transgenic tobacco plants overexpressing AtCGS (Hacham et al., 2002, 2006). To study the effect of enhanced flux towards lysine synthesis on methionine metabolism, we crossed homozygous tobacco plants overexpressing feedback-insensitive DHPS with homozygous transgenic tobacco plants overexpressing AtCGS to produce a new transgenic line that overexpresses both the bacterial DHPS and AtCGS (see Experimental procedures). Two separate crosses were performed, and as the results obtained from the progeny of these two crosses were very similar, the results presented below described only one set of crosses. For these crosses, we selected transgenic plants expressing AtCGS without morphological phenotype (Hacham et al., 2006). However, plants overexpressing bacterial DHPS exhibited the typical abnormal morphological phenotype, including mosaic green color in newly developed leaves at the tip of the apex and partial loss of apical dominance (Shaul and Galili, 1992a). When grown together with plants expressing the DHPS gene alone, the newly produced transgenic line, expressing both the AtCGS and DHPS genes, exhibited a weaker phenotype than DHPS plants (data not shown), the reason for which is not known.

The lysine branch competes with that of methionine/threonine for the carbon/amino skeleton 3-aspartic semialdehyde, originally derived from aspartate (Figure 1). As CGS is located downstream in the threonine/methionine branch, it was expected that the level of methionine would be reduced in the background of overexpression of bacterial DHPS, which enhances the flux of 3-aspartic semi-aldehyde towards lysine synthesis (Figure 1). Unexpectedly, however, methionine levels were significantly elevated in plants co-expressing both transgenes compared with those expressing AtCGS alone (Figure 2a). The level of SMM, the storage and mobile form of methionine, which is usually correlated with the soluble methionine content (Hacham et al., 2002), was also significantly increased in these plants (Figure 2a). Notably, the level of SMM also increased significantly in plants overexpressing the bacterial DHPS alone compared with wild-type plants. The level of lysine did not significantly differ in CGS/DHPS plants compared with those expressing only DHPS (Figure 2a).

The higher levels of lysine and methionine in plants expressing both transgenes were accompanied by a significant reduction in the level of aspartate, the precursor of the aspartate family pathway, and glutamine, which was reduced approximately fivefold relative to wild-type plants (Table 1). The levels of other amino acids did not differ significantly from those of the wild-type plants, including the

Figure 2. Amino acid contents in seeds and leaves of transgenic plants. (a) Methionine, S-methylmethionine (SMM) and lysine contents in leaves of transgenic plants and in control, wild-type (WT) tobacco plants. (b) Methionine and lysine contents in seeds of transgenic and wild-type plants. Extracts were obtained from the leaves of 12-week-old wild-type plants, or from seeds of transgenic plants overexpressing bacterial DHPS, Arabidopsis CGS, or both. The amounts of amino acids were calculated from total amino acids as detected by HPLC and are given in mol% of the total. The data presented represent the mean and SD of six plants per line. ANOVA was used to determine statistically significant differences ($P < 0.05$) as identified by different letters.
Table 1 Soluble amino acid contents in tobacco plants expressing Arabidopsis CGS (AtCGS), bacterial DHPS or both transgenes

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>(%)</th>
<th>Wild-type</th>
<th>AtCGS plants</th>
<th>DHPS plants</th>
<th>AtCGS/DHPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Met</td>
<td>0.3 ± 0.1</td>
<td>1.4 ± 0.3</td>
<td>0.6 ± 0.2</td>
<td>3.1 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>SMM</td>
<td>0.5 ± 0.1</td>
<td>2.3 ± 0.4</td>
<td>1.2 ± 0.2</td>
<td>3.6 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>Lys</td>
<td>1.2 ± 0.4</td>
<td>0.8 ± 0.3</td>
<td>30.0 ± 3.2</td>
<td>38.9 ± 4.2</td>
<td></td>
</tr>
<tr>
<td>Asp</td>
<td>8.5 ± 1.8</td>
<td>4.7 ± 1.7</td>
<td>6.2 ± 1.4</td>
<td>3.5 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>Thr</td>
<td>2.9 ± 0.2</td>
<td>4.3 ± 0.4</td>
<td>2.1 ± 0.1</td>
<td>3.1 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>Glu</td>
<td>22.8 ± 3.9</td>
<td>21.1 ± 2.8</td>
<td>15.8 ± 1.7</td>
<td>16.3 ± 5.9</td>
<td></td>
</tr>
<tr>
<td>Asn</td>
<td>2.4 ± 0.3</td>
<td>2.9 ± 0.9</td>
<td>3.1 ± 0.6</td>
<td>2.0 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>Ser</td>
<td>6.7 ± 0.1</td>
<td>7.2 ± 2.7</td>
<td>5.2 ± 0.9</td>
<td>5.7 ± 2.2</td>
<td></td>
</tr>
<tr>
<td>Gin</td>
<td>26.9 ± 3.3</td>
<td>24.5 ± 3.4</td>
<td>12.8 ± 4.8</td>
<td>5.4 ± 1.5</td>
<td></td>
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<tr>
<td>Gly</td>
<td>13.3 ± 2.4</td>
<td>12.8 ± 2.7</td>
<td>9.6 ± 2.1</td>
<td>13.1 ± 4.0</td>
<td></td>
</tr>
<tr>
<td>His</td>
<td>0.9 ± 0.1</td>
<td>1.0 ± 0.3</td>
<td>0.8 ± 0.2</td>
<td>1.1 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Arg</td>
<td>0.9 ± 0.3</td>
<td>1.3 ± 0.6</td>
<td>1.6 ± 0.4</td>
<td>1.2 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>5.6 ± 1.1</td>
<td>8.2 ± 3.1</td>
<td>4.6 ± 0.4</td>
<td>8.1 ± 4.1</td>
<td></td>
</tr>
<tr>
<td>Pro</td>
<td>2.6 ± 0.5</td>
<td>3.2 ± 0.6</td>
<td>2.9 ± 0.3</td>
<td>3.1 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>Tyr</td>
<td>0.4 ± 0.2</td>
<td>0.6 ± 0.2</td>
<td>0.8 ± 0.7</td>
<td>0.6 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Val</td>
<td>0.8 ± 0.1</td>
<td>0.9 ± 0.4</td>
<td>0.7 ± 0.1</td>
<td>0.6 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Ile</td>
<td>0.8 ± 0.3</td>
<td>1.3 ± 0.5</td>
<td>0.7 ± 0.1</td>
<td>1.3 ± 0.2</td>
<td></td>
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<tr>
<td>Leu</td>
<td>0.6 ± 0.1</td>
<td>0.8 ± 0.3</td>
<td>0.6 ± 0.1</td>
<td>0.7 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Phe</td>
<td>0.8 ± 0.3</td>
<td>0.8 ± 0.2</td>
<td>0.5 ± 0.1</td>
<td>0.5 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Trp</td>
<td>0.3 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.0</td>
<td>0.3 ± 0.1</td>
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</tr>
</tbody>
</table>

The amounts of amino acids were calculated from total amino acids as detected by HPLC and are given in mol% of this total. The total amino acid content did not differ significantly between wild-type and transgenic lines. Six plants of each type were analyzed and the data are presented as means ± SE. ANOVA was used to determine statistically significant differences (P < 0.05) as identified by different superscript letters.

Lysine affects the expression level of SAMS and methionine content

In transgenic tobacco plants, methionine content is positively correlated with the expression level of AtCGS (Hacham et al., 2006). In an attempt to account for the elevated levels of methionine and SMM in plants expressing both transgenes, we examined the expression of AtCGS by immunoblot and Northern blot analysis. The mRNA and protein expression levels of AtCGS in these CGS/DHPS plants were increased 5.5-fold compared with plants expressing AtCGS alone (Figure 3). These results demonstrate that overexpression of DHPS and the increased amount of lysine modulate the accumulation of AtCGS mRNA and protein. This higher amount of AtCGS probably accounts for the high-level accumulation of methionine and SMM in these plants.

Lysine application increases the amount of CGS in Arabidopsis plants

The results described so far suggest that lysine enhances the expression level of CGS. To substantiate this suggestion by level of threonine, which competes with methionine for the same carbon/amino skeleton (Table 1).

High levels of amino acids in leaves can influence the levels of these amino acids in seeds. Therefore, the levels of lysine and methionine were examined in seeds of these transgenic plants as well as in wild-type seeds. It was found that the methionine level does not differ significantly between seeds of transgenic and wild-type plants (Figure 2b). However, the lysine level was found to increase in plants expressing DHPS alone, but this elevation was not found in plants expressing both transgenes, AtCGS and DHPS (Figure 2b). These findings suggest that the marked increase in the levels of these amino acids in leaves does not necessarily affect their levels in seeds. A similar observation was also made in transgenic barley plants overexpressing the bacterial AK or DHPS genes, in which the levels of lysine and threonine significantly increased in leaves but not in seeds (Brinch-Pedersen et al., 1996).

CGS over-accumulates in CGS/DHPS plants

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The results described so far suggest that lysine enhances the expression level of CGS. To substantiate this suggestion by

![Figure 3](image)

(a) Immunoblot analysis of total soluble proteins using antibodies against the SHA epitope tag. Coomassie blue staining of the Rubisco band was used to determine equal loading.
(b) RNA blot analysis of total RNA isolated from leaves hybridized with Arabidopsis CGS cDNA as a probe. Methylene blue staining of 18S rRNA was used as an internal loading control.

Quantifications of band intensities (mean and SD) of six plants analyzed by Western blot and Northern blot analysis. The mRNA and protein expression levels of AtCGS in these CGS/DHPS plants were increased 5.5-fold compared with plants expressing AtCGS alone (Figure 3). These results demonstrate that overexpression of DHPS and the increased amount of lysine modulate the accumulation of AtCGS mRNA and protein. This higher amount of AtCGS probably accounts for the high-level accumulation of methionine and SMM in these plants.

(b) RNA blot analysis of total RNA isolated from leaves hybridized with Arabidopsis CGS cDNA as a probe. Methylene blue staining of 18S rRNA was used as an internal loading control.

Quantifications of band intensities (mean and SD) of six plants analyzed by Western blot and Northern blot, respectively, using the BioImage Intelligent Quantifier (Quantity One, Bio Rad, Hercules, CA, USA), are shown below the blots. Ratios were calculated comparing the band intensities in the Western analyses and Northern blots against those for the bands obtained for Coomassie blue and methylene blue staining, respectively. Statistically significant differences (P < 0.05, using Student’s t-test) are identified by asterisks.
another approach, wild-type Arabidopsis seedlings were irrigated with 2 mM of lysine for 24 h, and the accumulation of AtCGS protein and its transcript was compared with that in plants treated with water. The expression level of AtCGS transcript as well as that of the protein were significantly increased in plants irrigated with lysine (Figure 4).

In Arabidopsis, the amount of CGS mRNA is modulated by SAM in a mechanism involving translation arrest and regulation of RNA degradation (Onouchi et al., 2005). This type of modulation has been noted in an in vitro coupled transcription/translation system based on wheatgerm extract (Chiba et al., 2003; Kreft et al., 2003; Onouchi et al., 2005). To examine whether lysine directly modulates the amount of CGS transcript by a similar mechanism, we used the same system here. Thus, cDNA encoding AtCGS was transcribed/translated in the presence of 0.5 and 1 mM lysine, and in the presence of methionine as a control. The amount of CGS was analyzed by immunoblot assay (Figure 5a). A low concentration of 4 μM lysine or methionine was applied to the control sample because, as observed previously, protein synthesis is not limited at that concentration (Hacham et al., 2006). While methionine caused a reduction in the level of AtCGS, lysine did not affect the AtCGS level (Figure 5). Therefore, unlike methionine/SAM, lysine does not affect CGS accumulation in the in vitro transcription/translation system. These results suggest that the mechanism by which lysine enhances the expression level of CGS is not mediated through direct binding to the protein or its transcript. Therefore, additional components such as metabolites and/or proteins that are not present in the in vitro transcription/translation system are likely to be involved.

Lysine negatively affects the mRNA level of the four Arabidopsis SAMS genes

In Arabidopsis, the transcript and protein amounts of CGS are modulated in an opposite manner to those of SAMS, a family of enzymes that convert methionine to SAM.
(Figures 5 and 7 in Kim et al., 2002). This negative correlation between the expression of CGS and SAMS might be expected because an increase in the level of SAMS could lead to an increase in the amount of the product, SAM, which is a negative regulator of CGS expression (Chiba et al., 2003; Onouchi et al., 2005) (Figure 1). If lysine regulates the expression of SAMS (transcript and/or protein), then it will also indirectly affect that of CGS. To test this hypothesis, the protein level of SAMS was analyzed in Arabidopsis seedlings irrigated with 2 mM lysine. The results showed a nearly 2.3-fold decrease in the SAMS protein level compared with plants treated with water (Figure 6a). To test whether a similar effect also occurs at the transcript level, semi-quantitative RT-PCR was performed with oligonucleotides derived from the four Arabidopsis SAMS genes. The mRNA amounts of the four SAMS genes were significantly reduced (Figure 6b). Next, the transcript levels of SAMS were measured in tobacco plants expressing AtCGS/DHPS, where the level of CGS was significantly increased. Using semi-quantitative PCR, we found, as expected from the results obtained in Arabidopsis, that the level of SAMS was reduced in these plants compared with plants expressing only AtCGS (Figure 6c). A similar reduction in SAMS level was also obtained when leaves of transgenic tobacco plants overexpressing the AtCGS were infiltrated with 2 mM lysine for 6 h (Figure 6d). Such a reduction was not observed when the leaves were infiltrated with water.

![Figure 6](image-url)
Low expression of SAMS can affect the SAM level. Therefore, the SAM level was determined using LC-MS in transgenic tobacco leaves overexpressing AtCGS, which were infiltrated with 2 mM lysine, or with water or 2 mM methionine as a control, for 5 h. It was found that, while the SAM level almost doubled in leaves treated with methionine, it was significantly reduced in leaves treated with lysine (about 30% in comparison to leaves treated with water) (Figure 7). The reduction in SAM level in the presence of lysine could explain the higher expression level of AtCGS in AtCGS/DHPS transgenic plants and in Arabidopsis plants irrigated with lysine (Figure 4).

These results suggest that lysine negatively modulates the expression level of SAMS. This modulation could be performed either by direct binding of lysine to domain(s) found in the SAMS transcript, or indirectly by its effect on another metabolite or gene expression component(s) that, as a consequence, affects SAMS. To distinguish between these mechanisms and test whether lysine affects the expression level of SAMS by direct binding to the protein or transcript, we used the in vitro coupled transcription/translation system described above. cDNAs encoding SAMS1 and SAMS3 were chosen as representatives of this family and were transcribed/translated in the presence of 0.5 and 1 mM lysine. The results showed reductions of 45% and 71% in the protein amounts of SAMS1 in the presence of 0.5 and 1 mM of lysine, respectively (Figure 8). A similar but less pronounced effect was found for SAMS3. Taken together, the data obtained suggest that lysine directly modulates the transcript level of SAMS, leading to a reduction in the level of SAM.

Discussion

In order to study the cross-talk between the methionine and lysine biosynthesis pathways, we produced transgenic tobacco plants overexpressing the two key enzymes controlling lysine and methionine biosynthesis pathways, DHPS and CGS, respectively. We used tobacco plants expressing the bacterial DHPS gene, which, unlike the plant enzymes, is feedback-insensitive to lysine (Shaual and Galili, 1992a). Those transgenic plants that exhibited significantly higher levels of lysine were crossed with tobacco plants overexpressing the AtCGS gene. The latter plants contain significantly higher levels of methionine and its metabolite, SMM (Hacham et al., 2002, 2006).

As these key enzymes are located in competing branches of the aspartate family biosynthesis pathway, it was expected that the level of methionine would be reduced in plants expressing DHPS/AtCGS compared with those expressing AtCGS alone. However, unexpectedly, the levels of methionine and its metabolite, SMM, were significantly increased compared with plants expressing AtCGS alone. In these high-methionine plants, the expression level of CGS was increased, while that of SAMS was reduced. The reduction of SAMS affects the SAM level, which was significantly reduced in plants treated with lysine. In vitro studies conducted using the wheatgerm transcription/translation system demonstrated that lysine did not increase the CGS expression level directly, but rather reduced the amount of SAMS (Figures 5 and 7). The expression level of SAMS is negatively correlated with the expression level of CGS, as found in Arabidopsis (Kim et al., 2002), and a negative correlation may be expected as SAMS produces SAM, and SAM is a negative regulator of AtCGS expression (Chiba et al., 2003).

Taking into account these new results, we propose the following scheme for the cross-talk between the lysine and
Lysine affects the expression level of SAMS and methionine content

methionine biosynthesis pathways (Figure 1, dotted line): A high level of lysine brings about a reduction in the amount of the enzyme SAMS due to a reduction in the level of transcripts encoding this enzyme. This leads to a reduction in the amount of SAM, which negatively regulates the amount of CGS transcript (Chiba et al., 2003; Onouchi et al., 2005). As a result, the expression of CGS is increased, and consequently the level of methionine (Figure 1). The methionine level can also be enhanced from reduction of the flux towards SAM and its metabolites (Giovannelli et al., 1985; Ranocha et al., 2001). Taken together, this mechanism ensures a fine balance between the amounts of lysine and methionine through an indirect biochemical cross-talk mechanism involving regulation of the expression of SAMS.

The cross-talk scheme between lysine and SAMS has been determined by analyzing transgenic plants over-expressing bacterial DHPS and AtCGS, by in vitro transcription/translation assays, and by lysine feeding experiments. However, when considering wild-type plants, the question remain as to the conditions under which lysine accumulates to high levels and thus modulates the methionine content and its metabolites. A recent report has shown that elevated lysine levels are obtained in plants exposed to ABA and sugar starvation, as examples of hormonal and metabolic signals (Stepansky et al., 2005). Therefore, the high lysine content under such stress conditions can affect the level of SAM and its metabolites. In addition, the levels of soluble lysine are found to increase in young leaves and at shoot apices where it is required for protein synthesis (G. Galili, Weizmann Institute, Rechovot, Israel, personal communication). As methionine is generally converted rapidly to SAM and its other metabolites (Giovannelli et al., 1985; Ranocha et al., 2001), such an effect of lysine on the level of SAMS enzymes may reduce the conversion rate of methionine to SAM and increase the level of soluble methionine, which is required together with lysine for protein synthesis.

The increase of lysine and methionine in the tobacco leaves of CDS/DHPS plants was associated with reduced levels of aspartate and glutamine (Table 1). This finding is not surprising as aspartate is the precursor of the aspartate family biosynthesis pathway leading to lysine, threonine and methionine (Figure 1), and glutamine is the precursor of glutamate, which, together with asparagine, is a precursor of aspartate (Figure 1) (Coruzzi and Last, 2000). The results obtained in the current study suggest that high flux towards lysine and methionine biosynthesis may trigger the conversion of glutamine, via glutamate, to aspartate and aspartate metabolites. However, this regulation apparently does not take place in other plants or tissues, as the levels of glutamine and asparagine were not reduced and even increased in Arabidopsis seeds accumulating higher levels of lysine and methionine (Zhu and Galili, 2003, 2004). Therefore, differences in the level of aspartate family products can affect the level of amide amino acids as well as glutamate and aspartate, the four major amino acids in plants, but the availability of these amino acids is tissue-dependent and, most probably, plant species-dependent.

The level of threonine was not significantly altered in the CDS/DHPS plants compared with wild-type plants (Table 1). This finding was unexpected for two main reasons. First, assuming that the level of SAM is reduced in CDS/DHPS plants, this should lower the activity of TS (Curien et al., 1998) and therefore lead to a lower level of threonine. Second, the enhanced flux of the carbon/amino skeleton toward lysine and methionine synthesis should lead to reduction in threonine content. The latter mechanism is supported by several lines of evidence as explained below. Plants expressing only bacterial DHPS have a significantly
lower level of threonine (Ben-Tzvi-Tzchori et al., 1996; Zhu and Galili, 2003, 2004) (Table 1). As these two amino acids (lysine and threonine) compete for the aspartate metabolite, 3-aspartic semi-aldehyde, as a common substrate in their biosynthesis pathways, this reduction in threonine level was anticipated (Galili, 1995). In the CGS/DHPS plants, lysine content was the same as in DHPS plants, thus the threonine level was expected to be reduced. Moreover, in these plants, methionine, whose biosynthesis pathway diverges from threonine branch, was also significantly elevated, meaning that more OPH was channeled towards methionine synthesis. Therefore, due to the significant elevation in lysine and methionine, a significant reduction in threonine level in these plants was expected. However, the main difference between plants expressing only DHPS and those expressing both genes was the higher methionine content in the latter. Previously, it was observed that methionine enhanced the expression level of Arabidopsis TS, and plants irrigated with methionine demonstrated a higher level of threonine (Avraham and Amir, 2005). Therefore, one of the possible mechanisms for the non-reduced threonine content in CGS/DHPS plants is that the expression level of tobacco TS in these plants is enhanced by methionine, which leads to enhanced threonine synthesis. Alternatively, two other mechanisms could explain this observation. First, it is possible that the non-reduced threonine level in the CGS/DHPS plants originated from reduced flux towards methionine synthesis and its metabolites, leaving more OPH for threonine synthesis. If this is the case, it may be assumed that enhancement of methionine in these plants stemmed not from elevation in its synthesis but rather from reduction of its catabolism to SAM. Second, as the threonine level is regulated not only by the rate of its synthesis but also by the rate of its catabolism (Joshi et al., 2006), it may be assumed that, when the methionine and lysine levels are increased, the level of threonine catabolism is reduced due to lower expression of threonine aldolase and/or threonine deaminase. Indeed, an altered expression level of both of these enzymes leads to changes in threonine content in Arabidopsis (Joshi et al., 2006). In either case, further studies are required to elucidate regulation of the threonine content in the different transgenic lines.

From a biotechnological standpoint, the results obtained suggest a novel approach to improve the nutritional quality of crop plants by increasing the amounts of the nutritionally important amino acids lysine and methionine. Our results demonstrate that expression of a feedback-insensitive DHPS and AtCGS could constitute a feasible approach for increasing lysine and methionine in crop plants. Notably, in the CGS/DHPS transgenic plants, the level of threonine, the third most important essential amino acid of the aspartate family, was not reduced. Application of this approach is important mainly for cereals and legume grains, the main protein donor crops for human and domestic animals, which suffer from a low lysine/methionine/threonine content in their proteins (Galili et al., 2005).

Experimental procedures

Plant growth conditions

The Arabidopsis plants were grown in a controlled growth chamber (16 h light/8 h dark, at 22 ± 3°C). The tobacco plants were grown in another growth chamber under 14 h light and 10 h dark, at 27 ± 3°C.

Statistical analysis

The data obtained from this study were analyzed statistically using SPSS software adapted to Windows, version 12. Within this software, we used ANOVA and Student’s t-test programs as indicated.

Generation of transgenic plants expressing DHPS/AtCGS

Homozygous tobacco plants expressing bacterial feedback-insensitive DHPS (kindly donated by G. Galili) (Shaull and Galili, 1992a) were crossed with homozygous transgenic tobacco plants over-expressing full-length AtCGS (Hacham et al., 2002, 2006). The expression level of both transgenes was verified by Western blot analysis in the leaves of 12-week-old heterozygous F1 plants (not shown). Following self-pollination, 30 F2 plants were grown in the greenhouse. These F2 plants were divided into three classes: carriers of the AtCGS gene, carriers of bacterial DHPS, and those expressing both transgenes. Plants of all classes were heterozygous. Non-transformed wild-type plants were used as a control.

Analysis of amino acids

Samples from 12-week-old tobacco leaves were ground in liquid nitrogen and kept frozen. Free amino acids were extracted from a sample of frozen leaves, essentially as described by Bielecki and Turner (1996). Approximately 200 mg tissue was homogenized by mortar and pestle in the presence of 600 µl of water/chloroform:methanol (3:5:12 v/v). Following centrifugation (2 min, top speed), the supernatant was collected and the residue was re-extracted with 600 µl of the same mixture. The two supernatants were combined, chloroform (300 µl) and water (450 µl) were added, and the resulting mixture was re-centrifuged for 5 min at top-speed. The upper water–methanol phase was collected, dried, and dissolved in 200 µl water. The concentration of free amino acids was determined using O-phthalaldehyde reagent, followed by measurement of the fluorescence at 335/477 nm. The amino acid composition was determined by loading a 66 nmol of the samples onto an RP-HPLC. We used a Nova Pak C18 column (4 µm, 3.9 mm × 150 mm; Waters, Milford Massachusetts, USA) in a Hewlett-Packard liquid chromatograph 1090 with an automatic injection system (Hewlett-Packard Agilent Technologie, Waldbronn, Germany). The 0.14 µM sodium acetate buffer, pH 6.4, was replaced with a corresponding ammonium acetate buffer (Martí et al., 1994).

Immunoblot analysis

Twenty-day-old Arabidopsis rosette leaves or 12-week-old tobacco leaves were homogenized using a mortar and pestle in buffer containing 100 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT and...
Lysine affects the expression level of SAMS and methionine content

1 mM PMSF at 4°C. After 20 min of centrifugation (16 000 g at 4°C), the supernatant was collected. Protein samples (10–40 μg) were fractionated on 10% SDS–PAGE (Laemmli, 1970), and transferred to a PVDF membrane using a Bio-Rad protein trans-blot apparatus (http://www.bio-rad.com/). The membrane was blocked overnight at 4°C in 5% v/v non-fat dried milk, and then reacted for 2 h at room temperature with either commercial anti-HA monoclonal antibodies (Roche, Mannheim, Germany), CGS rabbit antisemur (Hacham et al., 2006) or SAMS chicken antiserum (kindly donated by J. Schroeder, Universitaet Freiburg, Freiburg, Germany). After incubation with the matching anti-IgG antibody conjugated to horseradish peroxidase under the same conditions, immunodetec- tion was performed using an enhanced chemiluminescence kit (Pierce, Rockford, IL, USA) in accordance with the manufacturer’s instructions.

**RNA extraction and Northern analysis**

Total RNA was extracted from frozen material using the Tri reagent (Sigma-Aldrich; http://www.sigmaaldrich.com/) according to the manufacturer’s instructions. RNA samples (20 μg) were subjected to electrophoresis in 1% agarose gel containing 2.2 M formaldehyde and 50 mM MOPS, pH 7.0, and transferred onto a nitrocellulose Hybond N membrane (Amersham; http://www6.amershambiosciences.com/). The blots were hybridized for 12 h at 65°C with probes labeled with α-32P dCTP using a Rediprime kit (Amersham). The probe was a SphI and EcoRI fragment of the Arabidopsis cgs (Hacham et al., 2002). The amount of 18S rRNA (used to determine equal loading) was visualized by staining the membrane with methylene blue in 0.5 M sodium acetate, pH 5.3. The intensity of the bands was quantified using the Bioimage Intelligent Quantifier (Quantity One, Bio Rad, Hercules, CA, USA).

**Semi-quantitative RT-PCR**

RNA samples were treated with 0.4 U of DNase (Ambion, Austin, USA) for 30 min. First-strand cDNA was synthesized at 42°C with 2 μg total RNA as the template, oligo(dT) primer (500 ng per reaction) and 10 U of AMV reverse transcriptase (Promega; http://www.promega.com/), in 20 μl reaction mixtures, according to the manufacturer’s instructions. Arabidopsis cgs was amplified using forward primer 5'-TGTCGTCAAGCTGAGATTAAAGCC and reverse primer 5'-CAGTGCAGCTTCAAGCCTGT. Arabidopsis SAMS1 (accession number M55077) was amplified using forward primer 5'-GTCTCAAAACCTTCCTCCT and reverse primer 5'-TGTGGAATGGAACGAGAAACA. Arabidopsis SAMS2 (accession number M33217) was amplified using forward primer 5'-GTCTGTTTCCCCTCTCTGCT and reverse primer 5'-TATTGTGGTACTGGATGATG. Arabidopsis SAMS3 (accession number NM 112618) was amplified using forward primer 5'-GTGACTAAGCTAACCACCA and reverse primer 5'-CTAGGTTCGAGAAAGTG. Arabidopsis SAMS4 (accession number NM 129243) was amplified using forward primer 5'-TCCTACCCCTGAGTTGAGAAAGG and reverse primer 5'-GGTGTTCTCTGCTAAGAAGGCC. Tobacco SAMS (AY445582) was amplified using forward primer 5'-ATGGAAGCTTGCACCAAGGTC and reverse primer 5'-ATCTACAAGCCCAAGTGACCATAG. The gene encoding actin (accession number AT5 G09810), used as an internal control to normalize samples of Arabidopsis and tobacco, was amplified using forward primer 5'-GTGCAATGGAACTGGAATG and reverse primer 5'-AGACAGAGATGATGAGTG. The PCR reaction was carried out in a total volume of 50 μl with 2.5 U of Taq polymerase (Sigma-Aldrich, Sigma, St. Louis, MO, USA), 250 μM dNTP, 250 mM primers and 1 μl of first-strand cDNA as the template. The thermo-

cycling conditions were: 95°C for 2 min, then 20 cycles of 95°C for 30 sec, 60°C for 1 min and 72°C for 1 min, then 72°C for 10 min. PCR products (10 μl) were loaded on a 1.5% TAE agarose gel. The ratio of the band intensity of target genes versus that of actin was calculated using the Biolmage Intelligent Quantifier.

**In vitro transcription/translation system**

The cDNA form of Arabidopsis CGS was amplified as previously described (Hacham et al., 2006). Arabidopsis SAMS1 cDNA was amplified using forward primer 5'-CATGGGACAGCTTTCTATT-CACATCTGAG and reverse primers 5'-ACCCGGGAGCTTGGAGTT-TGTTCCACTGTAG (contains a Smal site), Arabidopsis SAMS3 cDNA was amplified using forward primer 5'-CATGGAATCTTTTT-TGTTACATCTGT and reverse primers 5'-ACCCGGGAGCTTGACCGTTAGACTGTT (contains an Smal site). The cDNA forms of Arabidopsis CGS and SAMS were ligated into pBlueScript KS+ (Stratagene; http://www.stratagene.com/) containing a 3HA epitope tag via SacI and Smal restriction sites, and subjected to further analysis. In vitro transcription and translation of CGS cDNA or SAMS cDNA were performed using the TNT coupled wheatgerm system (Promega). Reaction mixtures (25 μl each) were prepared utilizing an amino acid mixture supplied by the manufacturer at a final concentration of 4 μM. Final concentrations of 0.5 and 1 mM lysine were adjusted by adding appropriate quantities of lysine separately to each reaction mixture. As a control, the respective cDNAs were expressed in the presence of 4 μM lysine. Following incubation at 30°C for 90 min, the reaction was stopped by freezing the samples in liquid nitrogen. CGS or SAMS protein levels were subsequently determined by Western blot analysis using anti-HA monoclonal antibodies.

**Feeding experiments**

Arabidopsis seedlings were grown on Nitsch media (Duchefa, Biochemie B.V., Haarlem, Netherlands) in a controlled growth chamber (16 h photoperiod at 22 ± 3°C) for 20 days. Feeding experiments were done by adding 50 μl of 2 mM lysine to each plant for 24 h. As a control, 50 μl of distilled water was added. Twenty-day-old plants were assembled into four groups of 10 plants each, and the samples were frozen in liquid nitrogen until use. Each group was subjected to Western blot and RT-PCR analyses.

**Analysis of SAM content using LC-MS**

The entire procedure was performed at 4°C in the dark. Leaf samples were ground in liquid nitrogen and kept frozen at −20°C. Then 150 μl of pre-cooled 0.0005% TEA (triethanolamine) at pH 7.2 were added to 100 mg of ground plant material. After 5 min of centrifugation at 10 000 g, the supernatant was collected and filtered in a 20 μm syringe filter. The extracts collected were analyzed using a Waters 2790 HPLC system (Waters, Milford, USA) equipped with a micro-
mass triple quadruple Quatro-Ultima LC-MS instrument (Micro-
Mass, Manchester, UK). A sample volume of 20 μl was injected into the HPLC using a loop injector at a mobile phase flow of 0.5 ml min⁻¹ into an electron spray ionization (ESI) interface in the positive (ESI+) mode. The mobile phase was 0.0005% TEA at pH 7.2. High selectivity of the compound was achieved using the multiple reaction monitoring (MRM) method. The SAM mother ion (m/z 399.2) was fragmented using argon at collision energy of 17 eV (electron voulte). The fragmented ion (m/z 250) was collected for quantification of the SAM. Conformation was achieved by spiking.
the samples with pure SAM (Fluka, Sigma-Aldrich, Buchs, Switzerland), which was added to the leaf extracts. For quantification, the peak areas for the samples were compared with a standard curve peak area of SAM concentration ranging from 1 parts per million (ppm) to 50 part per billion (ppb). The SAM level was calculated as nmol g\(^{-1}\) fresh weight (FW).

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