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Overexpression of mutated forms of aspartate kinase and cystathionine γ-synthase in tobacco leaves resulted in the high accumulation of methionine and threonine

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Summary

Methionine and threonine are two essential amino acids, the levels of which limit the nutritional quality of plants. Both amino acids diverge from the same branch of the aspartate family biosynthesis pathway; therefore, their biosynthesis pathways compete for the same carbon/amino substrate. To further elucidate the regulation of methionine biosynthesis and seek ways of increasing the levels of these two amino acids, we crossed transgenic tobacco plants overexpressing the bacterial feedback-insensitive aspartate kinase (bAK), containing a significantly higher threonine level, with plants overexpressing Arabidopsis cystathionine γ-synthase (AtCGS), the first unique enzyme of methionine biosynthesis. Plants co-expressing bAK and the full-length AtCGS (F-AtCGS) have significantly higher methionine and threonine levels compared with the levels found in wild-type plants, but the methionine level does not increase beyond that found in plants expressing F-AtCGS alone. This finding can be explained through the feedback inhibition regulation mediated by the methionine metabolite on the transcript level of AtCGS. To test this assumption, plants expressing bAK were crossed with plants expressing two mutated forms of AtCGS in which the domains responsible for the feedback regulation have been deleted. Indeed, significantly higher methionine contents and its metabolites levels accumulated in the newly produced plants, and the levels of threonine were also significantly higher than in the wild-type plants. The transcript level of the two mutated forms of AtCGS significantly increased when there was a high content of threonine in the plants, suggesting that threonine modulates, probably indirectly, the transcript level of AtCGS.

Keywords: methionine metabolism, threonine, aspartate family, cystathionine γ-synthase, aspartate kinase, biotechnology.

Introduction

Methionine and threonine are of great nutritional importance in animal feed and human food because of the limited levels of these essential amino acids in seeds and vegetative tissues of many crop plants (recently reviewed by Amir and Tabe, 2006; Azevedo *et al.*, 2006; Galili *et al.*, 2005; Hesse and Hoefgen, 2003). These essential amino acids are synthesized in plants from aspartate via the threonine branch of the aspartate family biosynthetic pathway (Figure 1). Molecular and biochemical studies have shown that the isozymes of aspartate kinase (AK), the enzyme catalyzing the first step of the aspartate family biosynthesis pathway, are feedback-inhibited by threonine and lysine, two products of this family. Although threonine negatively regulates the activity of AK-homoserine dehydrogenase (Curien *et al.*, 2005; Paris *et al.*, 2003), lysine negatively regulates the activity of monofunctional AK isozymes (Curien *et al.*, 2005; Tang *et al.*, 1997). In addition, lysine also inhibits the activity of the first enzyme of its own pathway, dihydrodipicolinate synthase (DHPS), whereas threonine inhibits the activity of monofunctional isozymes of homoserine dehydrogenase, which regulates the carbon/amino flux towards methionine, threonine and isoleucine synthesis (Figure 1) (Azevedo *et al.*, 2006; Galili, 1995).

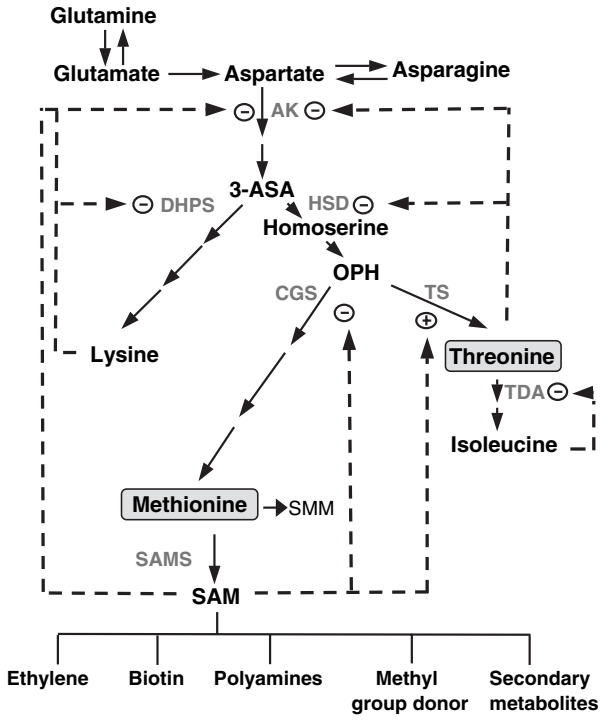

Figure 1. Scheme of the aspartate family biosynthesis pathway and methionine metabolism. Only some of the enzymes and metabolites are indicated. Abbreviations: AK, aspartate kinase; 3-ASA, 3-aspartic semialdehyde; CGS, cystathionine γ-synthase; DHPS, dihyrodipicolinate synthase; HSD, homoserine dehydrogenase; OPH, *O*-phosphohomoserine; SAM, *S*-adenosylmethionine; SMM, *S*-methylmethionine; SAMS, SAM synthase; TDA, threonine deaminase; TS, threonine synthase. Dashed arrows with a 'minus' sign represent the feedback inhibition loop on activities of key enzymes in the network; dashed arrows with a 'plus' sign represent the activation of the enzyme.

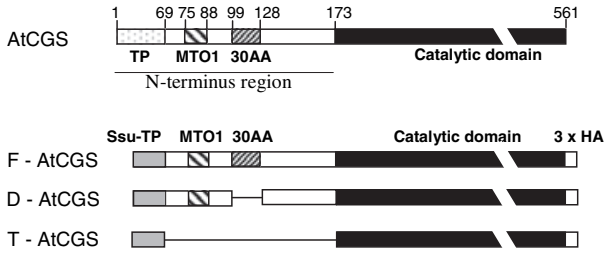

Figure 2. Upper part. Schematic presentation of the Arabidopsis cystathionine γ-synthase (AtCGS) protein. The amino acids are numbered from the first methionine codon of the protein in the transit peptide. Key: TP, the chloroplast-targeting transit peptide, which directs the protein into the chloroplast and is then removed; N-terminus, the N-terminus region of AtCGS (following the removal of the transit peptide), which shares no homology to CGSs of bacteria; MTO1, the MTO1 domain that is responsible for the AtCGS transcript degradation at a high level of *S*-adenosylmethionine (SAM); 30 AA, the 30–amino-acid domain the omission of which leads to the methionine insensitive form of AtCGS (D-AtCGS); catalytic region, the part of AtCGS sharing a homology to bacteria CGSs and harboring the catalytic site (T-AtCGS).Lower part. The constructs used to express the full-length (F-AtCGS), the truncated form lacking the N-terminus region (T-AtCGS) or the deleted form of CGS lacking the 30-AA domain (D-AtCGS). The transit peptide of the Rubisco small subunit 3A of pea (Ssu-TP) was used instead of the endogenous one. The sequence encoding three hemagglutinins (3 × HA) was added in frame at the C-termini of the proteins.

Molecular studies showed that AK is the major rate-limiting step of threonine biosynthesis *in vivo* (Frankard *et al.*, 1991; Galili, 1995; Shaul and Galili, 1992). Accordingly, a constitutive expression of the bacterial feedback-insensitive AK (bAK) in transgenic tobacco and alfalfa plants (Galili *et al.*, 2000; Shaul and Galili, 1992), or under a seed-specific promoter in tobacco plants (Karchi *et al.*, 1993), led to significant overproduction of threonine. However, the level of methionine, which diverges from this branch, was not significantly changed in leaves, and was only slightly increased in seeds (Galili *et al.*, 2000; Karchi *et al.*, 1993; Shaul and Galili, 1992).

The methionine level in Arabidopsis is regulated primarily by the level of cystathionine γ-synthase (CGS), which is the first and the key enzyme of the methionine biosynthetic pathway (Amir *et al.*, 2002; Avraham and Amir, 2005; Bartlem *et al.*, 2000; Hesse and Hoefgen, 2003; Kim *et al.*, 2002; Lee *et al.*, 2005; Onouchi *et al.*, 2005; Ravanel *et al.*, 1998; Zeh *et al.*, 2001). The mature plant CGS enzymes (without the plastid transit peptides), unlike bacterial CGS, contain an additional region of ~100 amino acids in the N-terminus that is not essential for the catalytic activity of this enzyme (Hacham *et al.*, 2002). To date, two subdomains have been identified in the N-terminus region of Arabidopsis CGS (AtCGS; Figure 2). The first, MTO1, a conserved region, is responsible for downregulating the mRNA level of AtCGS when the levels of the methionine metabolite, *S*-adenosylmethionine (SAM), increase (Chiba *et al.*, 2003; Onouchi *et al.*, 2004, 2005). The second domain is comprised of 90-nt (or 30 amino acids) located next to the MTO1 (Figure 2). A native form of AtCGS lacking this 90-nt domain was found to be insensitive to high methionine levels, even though it harbored the MTO1 domain (Hacham *et al.*, 2006).

Biochemical and molecular evidence demonstrated that the methionine level is also regulated concertedly by the level of threonine synthase (TS), the last enzyme in the threonine biosynthesis pathway, which competes with CGS for their common substrate, *O*-phosphohomoserine (OPH; Figure 1; 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; ; Zeh *et al.*, 2001; Ravanel *et al.*, 1998). *In vitro* biochemical studies showed that the affinity of TS to OPH is stimulated by SAM (Figure 1; Curien *et al.*, 1996; Curien *et al.*, 1998). This implies that methionine regulates its own synthesis through SAM. When a high level of methionine, and hence SAM, is produced, TS activity is increased (Curien *et al.*, 1996), causing reduced OPH availability for methionine synthesis. Under these conditions, *in vitro* activity measurements indicate that TS in plants has 250 to 500-fold higher affinity for OPH than does CGS,

causing reduced OPH availability for methionine synthesis (Curien *et al.*, 1998; Ravanel *et al.*, 1998b). However, a modeling analysis suggests that TS and AtCGS display similar kinetic efficiency for OPH, but that OPH is used more by TS because of the higher concentration of this enzyme compared with CGS (Curien *et al.*, 2003). Analyses of transgenic and mutant plants have also shown that the protein levels of TS and CGS are important for determining the distribution of OPH between the two pathways (reviewed by Amir *et al.*, 2002; Hesse and Hoefgen, 2003). The importance of the OPH level in methionine and threonine synthesis was recently shown in wild-type and transgenic Arabidopsis plants overexpressing homoserine kinase that were fed with homoserine, one metabolite upstream to OPH (Figure 1). The levels of both amino acids were significantly increased 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 AtCGS were fed with homoserine (Lee *et al.*, 2005). These results suggest that under physiological conditions, the CGS and TS are substrate limited, and that this probably occurs because of the close regulation of AK and homoserine dehydrogenase activities that limit OPH production. These results also indicate that a strategy to increase methionine might involve the co-expression of CGS, along with the feedback-insensitive mutant form of AK.

The objective of this research is to further explore the regulation of methionine synthesis, to study the factors regulating the level of AtCGS, and to gain more knowledge about the competition between methionine and threonine biosynthesis pathways. In addition, we also address the question of whether the level of methionine, as well as the levels of both methionine and threonine, can be enhanced in the same plant tissue, a question that is important in the biotechnological point of view. To this end, we crossed tobacco plants overexpressing bAK (Shaul and Galili, 1992) with those overexpressing three different forms of AtCGS (Hacham *et al.*, 2002, 2006). The results show that significantly higher methionine and threonine levels can be achieved in the same plant tissue. A significantly higher methionine level (up to a 176-fold increase) was obtained in plants overexpressing the bAK and methionine/SAM-insensitive forms of AtCGS when compared with wild-type plants. Notably, the threonine level was still significantly higher (39-fold) in these plants compared with the wild-type plants. In addition, the results suggest that threonine leads to the accumulation of the transcript level of AtCGS, although most probably indirectly. The results obtained in this study not only enhance our knowledge about the factors regulating methionine synthesis, but also suggest new ways of producing transgenic crop plants containing increased methionine and threonine levels, and hence having improved nutritional quality.

Results

Overexpressing bAK together with full-length AtCGS (F-AtCGS) did not lead to higher methionine content, compared with plants overexpressing only F-AtCGS

We previously obtained a significant accumulation of methionine, as well as its metabolite, *S*-methylmethionine (SMM), in transgenic tobacco plants overexpressing F-AtCGS (Hacham *et al.*, 2002, 2006). In our attempt to produce transgenic plants accumulating an even higher level of methionine, and to test the possibility of obtaining plants containing higher levels of methionine together with threonine, we crossed homozygous tobacco plants overexpressing F-AtCGS with homozygous plants overexpressing bAK (Shaul and Galili, 1992). The latter plants showed a significantly higher threonine level in their leaves (Shaul and Galili, 1992, 1993). As methionine biosynthesis diverges from the threonine branch, we hypothesized that significantly increasing the carbon/amino skeleton flux towards the threonine branch of the aspartate family would result in the overaccumulation of OPH, the substrate of CGS, and would therefore lead to a significant increase in methionine content.

For these crosses, we selected transgenic plants without morphological phenotypes. Two separate crosses were performed, and the expression levels of both alien genes were assayed from leaves of 15 separate 12-week-old heterozygous plants in each set of T_0 plants. Following self-pollination in each set, 40 T_1 plants from each set were grown in the greenhouse. These T_1 plants were divided into four classes: those carrying the *F-AtCGS* gene; those carrying the *bAK* gene; those expressing both foreign genes; and those carrying any of these foreign genes used as a control. As the results obtained from the progenies of these two crosses were very similar, the results presented below described only one set of crosses.

Plants co-expressing both foreign genes have significantly higher threonine and methionine levels when compared with levels in wild-type plants (Figure 3a; Table 1). However, the methionine level did not increase in plants co-expressing both foreign genes, compared with those expressing F-AtCGS alone, as was previously suggested by Lee *et al.* (2005). The threonine level was significantly reduced in plants expressing both foreign genes compared with those expressing bAK alone. Surprisingly, the level of isoleucine, the fourth amino acid belonging to the aspartate family, significantly increased in plants expressing both foreign genes by about sevenfold in comparison with the level found in wild-type plants (Table 1). The levels of other amino acids did not differ significantly between plants expressing *bAK* or *F-AtCGS* and those expressing both genes (Table 1).

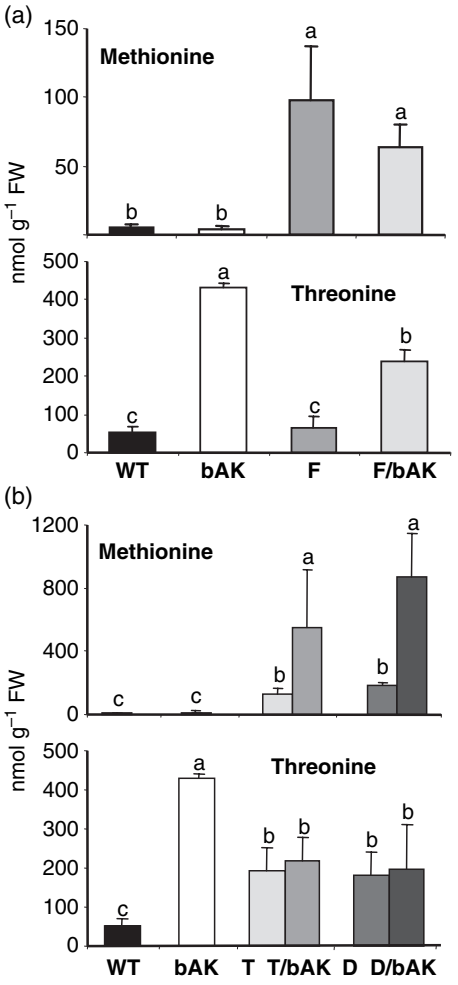

Figure 3. High accumulation of methionine and threonine levels in leaves of transgenic plants expressing: (a) bAK and F-AtCGS and (b) bAK together with T-AtCGS or D-AtCGS. Leaf extracts were obtained from 12-week-old wild-type plants (WT), transgenic plants expressing bacterial feedback-insensitive aspartate kinase (bAK), full-length Arabidopsis cystathionine γ-synthase (F-AtCGS) (F), both foreign enzymes (F/bAK), the truncated form of AtCGS (T-AtCGS) (T), both T-AtCGS and bAK (T/bAK), the deleted form of AtCGS (D-AtCGS) (D), and both D-AtCGS and bAK (D/bAK). The quantities of amino acids were analyzed using GC-MS, and their levels were calculated as nmol g^{-1} fresh weight of leaves. The data presented represent the mean ± standard deviation of six plants per line. The ANOVA test was used to determine statistically significant differences ($P < 0.05$) identified by different letters.

A significantly higher methionine level was found in plants expressing bAK and AtCGS without its N-terminus regulatory region (T-AtCGS)

In Arabidopsis, the methionine level is closely regulated by the expression level of AtCGS (Hacham *et al.*, 2006; Kim *et al.*, 2002), which is controlled by the level of the methionine metabolite, SAM (Chiba *et al.*, 2003; Onouchi *et al.*, 2005). One possible explanation for the observation that methionine was not further enhanced in plants expressing bAK/F-AtCGS genes, compared with plants expressing only F-AtCGS, is that the methionine and SAM levels reach a threshold in these plants, and that beyond this level the expression level of AtCGS begins to decrease as a result of SAM accumulation. To test this assumption, we crossed bAK plants with those expressing T-AtCGS. This form of AtCGS lacks its N-terminus region and therefore lacks the MTO1 domain (Hacham *et al.*, 2002; Figure 2).

Plants overexpressing T-AtCGS possess severe abnormal phenotypes that include flower sterility, and hence did not produce seeds (Hacham *et al.*, 2002). However, two transgenic plants out of a set of 44 that have a very low expression level of T-AtCGS produced seeds. Two progenies of these two plants were used for the crosses with two plants overexpressing bAK.

It was expected that plants expressing bAK and T-AtCGS would have a higher methionine level compared with plants expressing T-AtCGS alone. Indeed, as expected, it was found that in these plants, the methionine levels were 4–109-fold higher in comparison with plants expressing T-AtCGS alone or wild-type plants, respectively (Figure 3b). The threonine level decreased to about half that found in plants expressing bAK alone (Figure 3b). The levels of some of the other amino acids significantly increased in plants expressing T-AtCGS and in plants expressing both foreign genes (Table 1). These include aspartate, lysine and isoleucine, which belong to the aspartate family, leucine, valine, serine, glycine and the three aromatic amino acids (Table 1). Therefore, to further verify the rate of methionine accumulation in these plants, the level of methionine was also calculated as a mole percentage from the total amino acids measured (as described in Hacham *et al.*, 2007). It was shown that methionine increased in plants expressing both genes by 4.9- and 40-fold compared with plants expressing only T-AtCGS and wild-type plants, respectively. Taken together, these results suggest that methionine content is limited by the content of the carbon/amino skeleton, but this can barely be seen in plants expressing F-AtCGS because of the regulatory role of the MTO1 domain.

Methionine content was even further increased in plants expressing bAK and the deleted form of AtCGS (D-AtCGS)

We previously characterized an additional form of AtCGS in Arabidopsis plants, which has a deletion of 90 nt close to the MTO1 domain of CGS inside the N-terminus regulatory region of AtCGS (Hacham *et al.*, 2006; Figure 2). We called this version the deleted form of AtCGS (D-AtCGS). This 90-nt domain was involved in the regulation of CGS accumulation, because, unlike the expression of F-AtCGS, a high methionine level did not reduce the expression level of D-AtCGS (Hacham *et al.*, 2006). As a result, tobacco plants overexpressing D-AtCGS showed a significantly higher methionine level compared with plants overexpressing F-AtCGS

Table 1 Soluble amino acid contents in leaves of wild-type (WT) and transgenic tobacco plants expressing bAK, the full-length Arabidopsis CGS (F), the truncated form of AtCGS (T), the deleted form of AtCGS (D), or both, bAK and each of the three form of AtCGS genes (F × bAK; T × bAK; D × bAK)

(A)	WT	bAK	F	F × bAK	T	T × bAK	D	D × bAK
Met	4.9 ± 2.1^{d}	3.8 ± 2.6^{d}	98 ± 39.8^{bc}	64.2 ± 16.1^{c}	125.2 ± 41.5^{b}	545.1 ± 367.5^{a}	185.8 ± 18^{b}	866.3 ± 276.9^{a}
Thr	5.1 ± 18.2^{d}	430.3 ± 11.3^{a}	64.1 ± 29.4^{c}	237.4 ± 31.8^{b}	193.8 ± 49.3^{b}	217.8 ± 58.9^{b}	180.3 ± 9.2^{b}	197.7 ± 115.2^{b}
Asp	187.3 ± 79.5^{c}	184.7 ± 54.5^{c}	137.8 ± 62.5^{c}	111 ± 63.2^{c}	468.2 ± 190^{ab}	199 ± 112.2^{c}	760.2 ± 219^{a}	447.6 ± 79.6^{b}
Glu	117.3 ± 88.9^{c}	113.5 ± 57.2^{c}	142.9 ± 40.4^{c}	205.8 ± 71^{c}	658 ± 178.3^{a}	340.8 ± 14.3^{b}	697 ± 365^{a}	163.5 ± 100.4^{c}
Lys	3.2 ± 0.8^{b}	4.7 ± 0.8^{b}	3.3 ± 1.4^{b}	4.4 ± 1.8^{b}	29.6 ± 16^{a}	18.3 ± 8.9^{a}	12.9 ± 8.3^{a}	14.2 ± 6.4^{a}
Ile	5.6 ± 2.3^{c}	11.4 ± 3.2^{cb}	18.3 ± 8.8^{cb}	40.8 ± 24.2^{b}	91.6 ± 46^{a}	138.4 ± 44^{a}	31.8 ± 21^{b}	59.36 ± 40^{b}
Leu	10.6 ± 4.1^{b}	12.5 ± 2.9^{b}	16.7 ± 4.6^{b}	30.7 ± 20^{b}	78.6 ± 13.4^{a}	56.6 ± 23^{a}	56.7 ± 27^{a}	68.9 ± 8.3^{a}
Val	14.5 ± 5.7^{bc}	17.9 ± 4.9^{bc}	17.7 ± 6^{bc}	12.5 ± 4^{c}	53.1 ± 10.6^{a}	37.1 ± 10^{a}	39.2 ± 14.7^{a}	22.9 ± 3^{b}
Ser	115.7 ± 38^{b}	120.3 ± 8.4^{b}	137 ± 53.7^{b}	117 ± 30^{b}	355 ± 150^{a}	341.9 ± 30^{a}	274.3 ± 100^{ab}	192 ± 56^{ab}
Gly	179 ± 100	178 ± 116	175.2 ± 101	116.1 ± 24.8	205.2 ± 104	161.2 ± 22.8	222.7 ± 57.8	334.2 ± 143
Ala	66.4 ± 12.4	63.2 ± 11.4	63.6 ± 15.3	52 ± 11.5	83.4 ± 24.7	43.1 ± 8.8	107.5 ± 38.5	66.9 ± 24
Pro	19.6 ± 5.5	17.5 ± 5.7	30.8 ± 12.2	29.5 ± 8.2	35.1 ± 15.1	34.2 ± 13.2	27.8 ± 15	35.3 ± 14.7
Tyr	4.8 ± 1.5^{c}	5.1 ± 2.8^{c}	6.5 ± 2.7^{c}	7.5 ± 2.8^{c}	66.6 ± 28.6^{a}	61.1 ± 28.7^{a}	28.3 ± 14.2^{b}	22.4 ± 15^{b}
Phe	17.2 ± 4.9^{b}	26.2 ± 15^{b}	28.3 ± 13.8^{b}	40.8 ± 18.4^{ab}	81.6 ± 21^{a}	79.7 ± 35^{a}	33.6 ± 16^{ab}	33.1 ± 13^{ab}
Trp	0.8 ± 0.5^{c}	1.2 ± 0.5^{c}	1.7 ± 1^{c}	4.5 ± 1.6^{b}	20.6 ± 20^{a}	25 ± 5.4^{a}	7 ± 3.7^{b}	5.5 ± 3.2^{b}

The quantities of amino acids were calculated as nmol g^{-1} fresh weight, as detected by GC-MS. The data are presented as the mean ± SE obtained from six independent measurements. The ANOVA test was used to determine statistically significant differences ($P < 0.05$), which are identified by different letters.

(Hacham *et al.*, 2006). However, contrary to the situation of plants expressing T-AtCGS lacking the whole N-terminus domain described above, only a small phenotype was observed in plants expressing this deleted form, which was very similar to phenotypes exhibiting high expression levels of F-AtCGS (Hacham *et al.*, 2006). However, for crosses with bAK plants, we selected transgenic plants without morphological phenotypes. Next, we tested if the methionine level would be increased in plants co-expressing bAK and D-AtCGS. Plants co-expressing these genes were found to have significantly higher levels of methionine, which increased by about 4.6- and 176-fold in comparison with the levels found in transgenic plants expressing D-AtCGS alone and wild-type plants, respectively (Figure 3b). As the level of other amino acids increased in these plants, as observed for plants expressing T-AtCGS with or without bAK, the level of methionine was also calculated based on mole percentage. The results showed that the methionine level increased by 4.8-fold in plants expressing both genes compared with plants expressing the D-AtCGS alone, and 59-fold compared with the level in wild-type plants. The threonine level decreased to about half of that found in plants expressing only bAK, but was still significantly higher (about 39-fold) than the level found in wild-type plants (Figure 3b; Table 1). The reduction in threonine level suggests that higher levels of methionine found in these plants are apparently at the expense of threonine. Notably, the high methionine and threonine levels did not lead to a decrease in the third amino acid that belongs to the aspartate family, lysine, the level of which increased by about 3.5-fold when compared with wild-type plants. As observed in plants expressing bAK/T-AtCGS, the level of isoleucine also increased significantly in these plants in comparison with the level found in wild-type plants. Similarly, the levels of some of the other amino acids also increased in these plants (Table 1). Taken together, these experiments showed that plants can greatly enhance the accumulation of both methionine and threonine without any further abnormal phenotype by co-expressing bAK and D-AtCGS.

High methionine levels in leaves increased protein-bound methionine and affected the level of soluble methionine in seeds

The methionine level significantly increased in plants expressing T-AtCGS/bAK and in those expressing D-AtCGS/bAK compared with the wild-type plants. These high levels can affect the methionine level incorporated into the soluble leaves proteins (Hacham *et al.*, 2002), as well as the level of soluble methionine in the seeds of these plants. Therefore, we next measured the methionine level in both these tissues, as well as the level of threonine. To this end, the water-soluble proteins, representing about 80% of leaf proteins (Galili *et al.*, 2000), were extracted and then subjected to hydrolyzation. An analysis of amino acids was performed using GC-MS. As expected, the methionine level incorporated into proteins increased in the transgenic plants that exhibited a higher level of soluble methionine than in wild-type plants (Figure 4a). However, among the plants expressing both foreign genes, only bAK/T-AtCGS plants exhibited a high methionine level compared with their parental line, T-AtCGS.

The high methionine level in leaves also affects the soluble methionine in seeds, especially in plants expressing

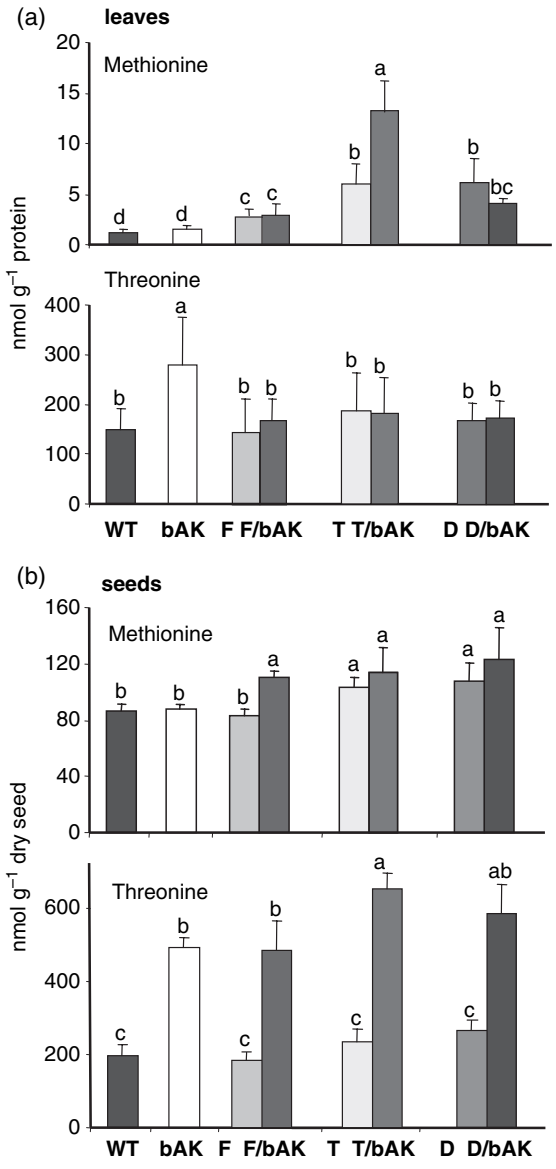

Figure 4. (a) The levels of methionine and threonine incorporated into the soluble proteins fraction of the leaves.
(b) Soluble methionine and threonine contents in dry seeds of transgenic and wild-type plants. Leaf extracts were obtained from 12-week-old plants or from dry seeds of wild-type (WT) and transgenic plants expressing bacterial feedback-insensitive aspartate kinase (bAK), full-length Arabidopsis cystathionine γ-synthase (F-AtCGS) (F), both F-AtCGS and bAK (F/bAK), the truncated form of AtCGS (T-AtCGS), both T-AtCGS and bAK (T/bAK), the deleted form of AtCGS (D-AtCGS), and both D-AtCGS and bAK (D/bAK). The amino acid levels incorporated into proteins were calculated from PBS-soluble proteins that were subjected to amino acid analysis following protein hydrolysis. The levels of these amino acids were analyzed by GC-MS. The data presented represent the mean ± standard deviation of six plants per line. The ANOVA test was used to determine statistically significant differences ($P < 0.05$) identified by different letters.

bAK together with each of the three forms of AtCGS. Slight but significantly increased methionine contents in seeds (about 25% compared with wild-type seeds) were observed. The results indicate that the methionine mobility towards seeds, or its level in seeds, is closely regulated when compared with the threonine level, as high levels of threonine in leaves significantly increases the threonine level in seeds by more than threefold (Figure 4b).

High levels of methionine metabolites, SMM, SAM, and dimethylsulfide (DMS), were found in plants that exhibit higher levels of methionine in leaves

We have previously shown that a high level of methionine can be positively correlated with the level of its metabolites (Hacham *et al.*, 2002, 2006, 2007). These include SMM, the storage and mobile form of methionine, SAM, its first metabolite, and DMS, the catabolic product of methionine. To gain more knowledge about methionine metabolism under conditions of high soluble methionine content in leaves, we measured the levels of these methionine metabolites. The results (Figure 5) demonstrated that the levels of SMM, SAM, and DMS, although increased in plants expressing F-AtCGS compared with wild-type plants, do not differ significantly from those expressing both foreign genes, F-AtCGS/bAK. These results imply that indeed, the methionine level does not increase in plants overexpressing F-AtCGS/bAK beyond that found in F-AtCGS plants. However, this was not the case in the transgenic plants expressing the T and D forms of AtCGS together with the bAK gene. In these plants, the methionine level increased significantly compared with plants expressing only the AtCGS forms, and the levels of SMM, SAM, and DMS increased in accordance (Figure 5). Notably, the levels of DMS and SAM increased in T-AtCGS/bAK plants more than in D-AtCGS/bAK plants, especially when compared with the increase of methionine in these plants.

Threonine application enhanced the expression levels of the T and D forms of AtCGS, but not the expression level of F-AtCGS

We previously found a positive correlation between methionine content and the expression level of AtCGS in transgenic tobacco plants (Hacham *et al.*, 2006). Hence, in an attempt to account for the significantly increased methionine and its metabolites levels in plants expressing the T and D forms of AtCGS, we examined the expression levels of the three forms of AtCGS in the transformed plants. The mRNA and protein expression levels of the T and D forms of AtCGSs in transgenic tobacco plants expressing these forms together with bAK were significantly higher, compared with plants expressing these two forms alone (Figure 6). Such an increase in AtCGS was not detected in plants expressing F-AtCGS/bAK (Figure 6).

As the threonine level (as calculated as mol%) was about 2-fold higher in plants expressing bAK together with the

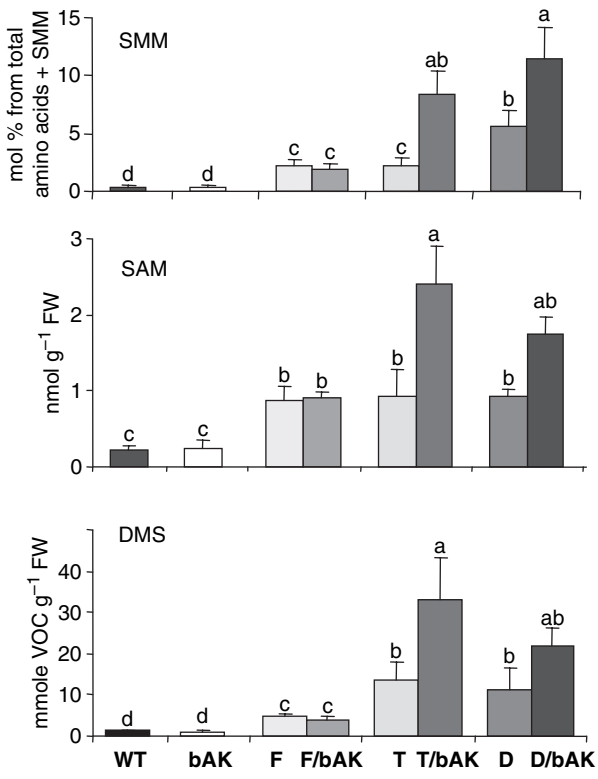

Figure 5. The levels of methionine metabolites, *S*-methylmethionine (SMM), *S*-adenosylmethionine (SAM) and dimethylsulfide (DMS), in leaves obtained from 12-week-old wild-type plants (WT), and transgenic plants expressing: (a) bacterial feedback-insensitive aspartate kinase (bAK) and full-length Arabidopsis cystathionine γ-synthase (F-AtCGS) (F) or both foreign enzymes (F/bAK); and (b) the bAK and truncated form of AtCGS (T-AtCGS) (T), both T-AtCGS and bAK (T/bAK), the deleted form of AtCGS (D-AtCGS) (D), and both D-AtCGS and bAK (D/bAK). The levels of SMM were calculated from total amino acids, as detected by HPLC, and are given in mole percentages (% mol) of the total of amino acids. The quantity of SAM was detected by LC-MS and expressed as nmol g^{-1} fresh weight of leaves. The level of DMS, a volatile organic compound (VOC), was measured by the purge-and-trap system connected to the GC-MS. The quantity of DMS was calculated by determining the area of the corresponding peak in the GC-MS graph compared with a known standard. The data presented represent the mean ± standard deviation of six plants per line. The ANOVA test was used to determine statistically significant differences ($P < 0.05$) identified by different letters.

T and D forms of AtCGS compared with plants overexpressing the T and D forms alone, the possibility was raised that threonine enhances the expression level of these forms. To examine this hypothesis, we transiently expressed the F, T, or D forms of AtCGSs in leaves of *Nicotiana betamiana*, and 48-h later, when high expressions of each type of AtCGS form were detected, threonine (2 mM) was injected into the same spots. As a control, water or methionine (2 mM) was injected into the other expressed AtCGS spots, and the expression levels of the three forms of AtCGS were examined 6-h later. Although methionine application leads to a reduction in the F-AtCGS expression level, unlike the T and D forms of AtCGS, the expression levels of the two latter forms of AtCGS were significantly increased by threonine application, but this increase was not observed in those expressing F-AtCGS (Figure 7).

Threonine could affect the transcript level of AtCGS directly, or alternatively, the modulation of the transcript level could be achieved indirectly as a result of the effect of threonine on yet unknown metabolites that affect the level of AtCGS. To distinguish between these two possibilities, we used the *in vitro* coupled transcription/translation system based on wheatgerm extract. This system was previously found to be reliable for studying the effect of methionine on the expression level of AtCGS (Chiba *et al.*, 2003; Hacham *et al.*, 2006, 2007; Kreft *et al.*, 2003; Onouchi *et al.*, 2005). Therefore, modulation of the expression level of the three forms of AtCGS in this *in vitro* system could point to a direct effect of threonine. The results presented in Figure 8 clearly demonstrate that although, as previously reported, methionine reduced the level of F-AtCGS (but not of D-AtCGS and T-AtCGS), the expression levels of the three forms of AtCGS were not affected by threonine application. Therefore, the modulation of T-AtCGS and D-AtCGS expression levels by threonine seems to be an indirect effect.

Discussion

To study the factors regulating methionine content, the expression level of AtCGS, and the diversion between methionine and threonine biosynthesis pathways, we produced transgenic tobacco plants overexpressing the mutated forms of two key enzymes of the threonine and methionine pathways, bAK and F-AtCGS, respectively. These plants also enabled us to address the question of whether the levels of methionine and threonine, two essential amino acids, can be increased in the same plant tissue. We assumed that a combination of bAK, which enhanced the flux of the carbon/amino skeleton towards the threonine branch of the aspartate family (Shaul and Galili, 1992), together with the overexpression of F-AtCGS (Hacham *et al.*, 2002), would lead to higher methionine and threonine levels in the same plant cells. A recent finding supports this assumption, because the levels of both methionine and threonine significantly increase in transgenic Arabidopsis plants overexpressing F-AtCGS fed with homoserine, one metabolite upstream to OPH (Lee *et al.*, 2005). Although, as expected, the levels of these two amino acids significantly increased in the same plant tissue compared with wild-type plants, the methionine levels, as well as its metabolites SMM, SAM and DMS, did not differ significantly from those found in its parent, i.e. plants expressing F-AtCGS alone (Figure 3; Figure 5). In addition, no significant differences were observed in the methionine that was incorporated into leaf proteins, and only a slight increase was found in the methionine content in seeds (Figure 4). These results suggest that the methionine level in plant cells and the expression level of F-AtCGS are below their

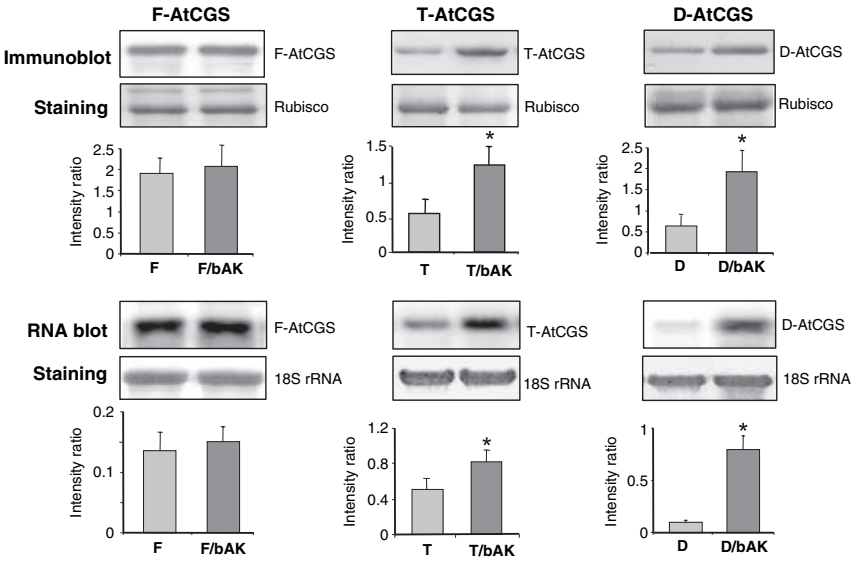

Figure 6. Higher accumulation of the truncated form of the Arabidopsis cystathionine γ-synthase (T—AtCGS) and the deleted form of AtCGS (D-AtCGS), unlike the full-length AtCGS (F-AtCGS), in plants expressing one of these forms of AtCGS together with bacterial feedback-insensitive aspartate kinase (bAK). Upper panels: immunoblot analysis using antibodies against the 3*HA epitope tag. Coomassie-blue staining of the rubisco band was used for equal loading. Lower panels: RNA blot analysis of total RNA isolated from leaves hybridized with AtCGS cDNA as a probe. Methylene-blue staining of the 18S rRNA was used as an internal loading control. Quantification of the band intensity of mean ± standard deviation of six plants analyzed by western and northern blots, respectively, using the Bioimage Intelligent Quantifier. The ratios between band intensities in the western and northern blots were calculated against the bands obtained in the coomassie-blue and methylene-blue staining, respectively. Statistically significant changes ($P < 0.05$, using the Student's *t*-test) are identified by asterisks.

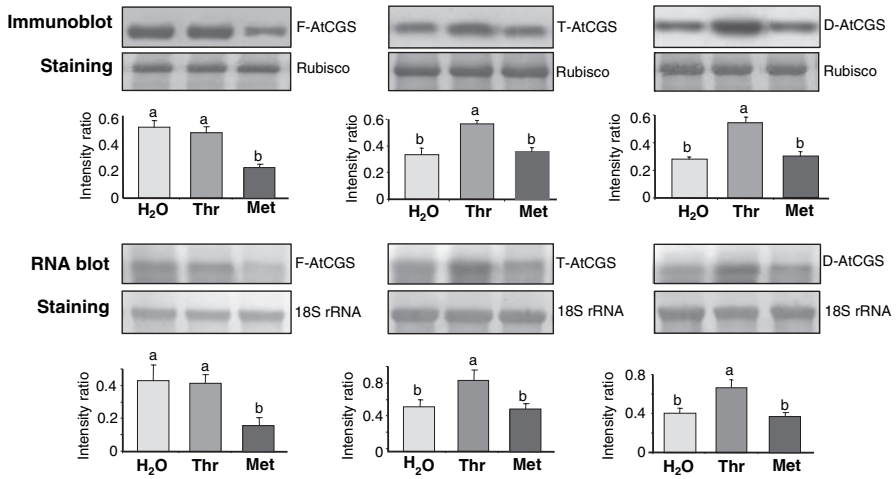

Figure 7. Threonine application enhanced the expression levels of the truncated (T) and deleted (D) forms of Arabidopsis cystathionine γ-synthase (AtCGS), but not the full-length form (F-AtCGS). Threonine, methionine (2 mM) or distilled water was infiltrated to leaves of *Nicotiana betamiana* after 48 h of *Agrobacterium* infiltration to the same spot, to achieve the transient expression of each of the three forms of AtCGS. Immunoblot analyses were performed using antibodies against 3HA epitope tag. The quantification of band intensity of mean ± standard deviation of five leaf spots was analyzed by western and northern blots, respectively, using the Bioimage Intelligent Quantifier. The ratios between band intensities in the western and northern blots were calculated against the bands obtained in the coomassie-blue and methylene-blue staining, respectively. The ANOVA test was used to determine statistically significant differences ($P < 0.05$) identified by different letters.

maximum values. Overexpression of F-AtCGS leads to an increase in methionine and hence an increase in SAM, which, beyond a certain threshold, leads to the mRNA degradation of F-AtCGS (Chiba *et al.*, 2003; Onouchi *et al.*, 2005). Therefore, the results presented in this study imply that overexpression of F-AtCGS alone already leads to high SAM production (Figure 5), which eventually leads to a degradation of the transcript of F-AtCGS, keeping the steady-state of the F-AtCGS transcript under tight control. Therefore, the excess in the carbon/amino skeleton derived from aspartate

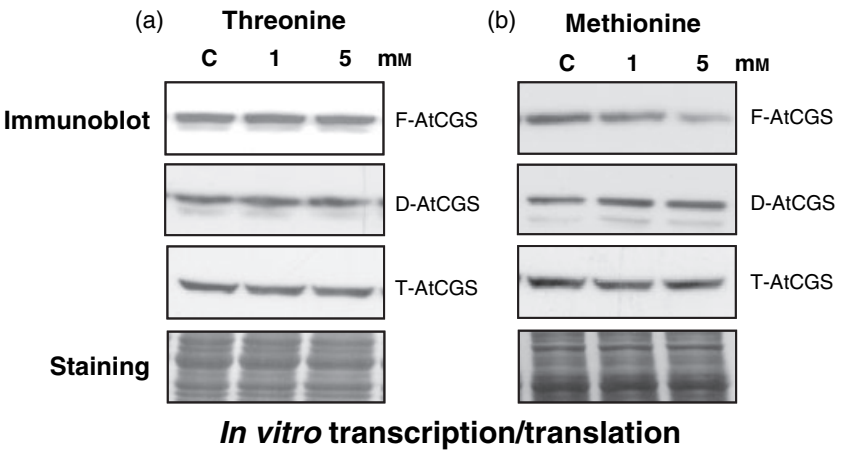

Figure 8. The expression level of Arabidopsis cystathionine γ-synthase (AtCGS) did not differ in a wheatgerm *in vitro* transcription/translation system in the presence of different concentrations of threonine (a), as opposed to the effect of methionine (b), which was used as a control. Samples containing 1 μg of linearized plasmid DNA (pBluescript KS$^-$) harboring cDNA encoding AtCGS were incubated in wheatgerm extracts containing 0.004 (C for control), 0.5 or 1 mM threonine (or methionine) for 90 min at 30°C. The quantity of AtCGS protein was determined by immunoblot assay.

under these conditions does not lead to a further increase in methionine content, because of this feedback inhibition mechanism.

To test this assumption, and to investigate whether feedback inhibition on the transcript level of F-AtCGS, mediated by the regulatory domains located inside the N-terminus region of AtCGS, plays a role in controlling the methionine level when the carbon/amino skeleton flux increases, we crossed plants overexpressing bAK and those expressing either T-AtCGS or D-AtCGS. These two forms of AtCGS are methionine insensitive because the T-AtCGS lacks its N-terminus region, and thus the MTO1 and 90-nt domains, whereas the D-AtCGS lacks the 90-nt domain alone. Plants expressing bAK with these two forms of AtCGS exhibit higher methionine levels, as well as methionine metabolites SMM, SAM and DMS, over plants expressing only the T or D forms of AtCGS (Figure 5; Table 1). The levels of methionine increased in plants expressing bAK/D-AtCGS to about 176-fold, in comparison with wild-type plants, suggesting a synergistic effect between the enhanced flux of the carbon/amino skeleton obtained by the expression of bAK, and the increased expression of this form of AtCGS. Taken together, these results showed that methionine content in plants cells can increase when the regulatory domains of AtCGS are omitted. Under such conditions, an increase in the carbon/amino skeleton flux led to a significant increase in methionine synthesis, and thus in its content.

The increase in methionine levels in the tobacco leaves of the T and D forms of AtCGS, and in plants also expressing these genes together with bAK, was associated with enhanced levels of most amino acid contents. The reason for this increase is not yet clear. However, this increase implies that the content of methionine (or one of its associated metabolites) beyond a certain threshold may serve as a signal, and thus, in plants with a higher level of methionine, it affects other biosynthesis pathways. Among the amino acids in which levels were increased were aspartate, the donor for the carbon/amino skeleton for methionine and threonine synthesis, and lysine, which belongs to the aspartate family. This suggests that methionine modulates the flux of the carbon/amino skeleton towards the aspartate family, as the level of members of this family increased in plants expressing the T and D forms of AtCGS, which have higher methionine levels. Surprisingly, the level of isoleucine, the fourth amino acid belonging to the aspartate family, also significantly increased in these plants (as well as in plants expressing bAK/F-AtCGS) in comparison with the level found in wild-type plants. Isoleucine is an essential branched-chain amino acid usually synthesized from threonine by threonine deaminase (Coruzzi and Last, 2000). Tobacco plants expressing the highest level of bAK showed a slight but significant increase in isoleucine level (Shaul and Galili, 1992). However, the isoleucine level is closely regulated by the activity of threonine deaminase, which is regulated by feedback inhibition, mediated by isoleucine (Wessel *et al.*, 2000). The higher levels of isoleucine found in plants co-expressing bAK and the T-AtCGS may also result in methionine degradation, as was recently found by Rebeille *et al.* (2006). The levels of valine, the biosynthesis pathway of which shares four enzymes with the isoleucine biosynthesis (Coruzzi and Last, 2000), and of leucine, which diverges from the valine biosynthesis pathway (Coruzzi and Last, 2000), were significantly increased in plants expressing the T and D forms of AtCGS alone or together with bAK (Table 1). Additional studies are required to reveal the relationships between methionine and the biosynthesis pathways resulting in isolecucine, valine and leucine.

The high methionine and methionine metabolite levels found in plants expressing bAK/D-AtCGS and bAK/T-AtCGS are most probably the result of the enhanced flux of the carbon/amino skeleton towards methionine synthesis, coupled with the high expression level of the two forms of AtCGS in these plants (Figure 7). We assume that threonine, the level of which increased 2-fold in these plants compared with the level in plants expressing each of these two forms

of AtCGS alone (based on mol%), leads to a high accumulation of the mRNA level of AtCGS. However, threonine most probably affects the level of AtCGS indirectly, as predicated from the *in vitro* studies (Figure 8). The 'threonine effect' can easily be detected in plants expressing bAK/D-AtCGS and bAK/T-AtCGS (Figure 6), but not in plants expressing F-AtCGS. These results lead us to hypothesize that two regulatory domains exist in the transcript of F-AtCGS. One is comprised of the MTO1/90-nt domain located in the N-terminal region of AtCGS, which is sensitive to SAM/methionine content, and the second domain is affected by threonine. As the effect of threonine was detected in the T-form of AtCGS, we suggest that this latter domain is not found at the N-terminus of AtCGS but is rather to be found in the catalytic region of AtCGS (Figure 2). As the catalytic domain is also found in F-AtCGS, the question still remains as to why the 'threonine effect' is not detected in plants overexpressing F-AtCGS/bAK. One possible answer to this question is that the effect of SAM/methionine on the accumulation of the F-AtCGS transcript is stronger than the 'threonine effect'. Therefore, the 'threonine effect' cannot be observed in F-AtCGS plants when a high level of threonine exists together with a high level of methionine. According to this assumption, the 'threonine effect' should be observed in the F-AtCGS transcript level when the methionine level is low and the threonine content is high. Indeed, it was previously reported that the expression level of the endogenous AtCGS in transgenic Arabidopsis plants increased significantly in plants with a higher threonine level, because of the overexpression of the bacterial or Arabidopsis threonine synthase (Avraham and Amir, 2005; Lee *et al.*, 2005). Moreover, feeding experiments with threonine led to a significant increase in the endogenous AtCGS content in Arabidopsis plants (Avraham and Amir, 2005). This finding supports the hypothesis that threonine affects the transcript level of F-AtCGS in Arabidopsis or transgenic tobacco plants, but that this effect can not be detected when the level of methionine is high in these plants. How threonine affects the transcript level of AtCGS is still an open question, and hence further studies are required to elucidate this point.

The results of the present study also suggest a new function for the D-form of AtCGS. This native form is found in Arabidopsis in all of the organs tested under normal growth conditions (Hacham *et al.*, 2006). As the transcript level of D-AtCGS increased when a high threonine level was found in plants, and as methionine and threonine compete for the same carbon/amino skeleton substrate (Figure 1), we suggest that the function of D-AtCGS is to attract more of this carbon/amino skeleton towards methionine synthesis when the threonine level increases, to keep the level of methionine high enough to support the methionine metabolism. This is an important role for D-AtCGS, because the methionine metabolites are essential for plant growth and development.

All in all, the results of the present study suggest that threonine indirectly leads to the accumulation of the transcript level of AtCGS, but that this effect is weaker than the effect of SAM. From a biotechnological standpoint, the results obtained in the current study suggest a novel approach to improve the nutritional quality of crop plants, by increasing the levels of nutritionally important essential amino acids, methionine and threonine, by expressing bAK and F-AtCGS. However, a significantly higher methionine level could be achieved in plants expressing bAK together with D-AtCGS. In these plants, the threonine level was still significantly higher in comparison with wild-type plants, and the lysine and isoleucine levels were significantly increased when compared with wild-type plants. As plants differ in their methionine regulation (Ravanel *et al.*, 1998; Hofgen *et al.*, 2001), such a manipulation using bAK and D-AtCGS could lead to differences in threonine or methionine content between various transgenic crop plants. This type of manipulation is important primarily for cereals and legume grains, the main protein donor crops for humans and domestic animals suffering from low levels of methionine, threonine and lysine in their proteins (Galili *et al.*, 2005).

Experimental procedures

Plant growth conditions

The tobacco plants were grown in another growth chamber with 14-h light and 10-h dark at 27 $\pm$ 3°C.

Statistical analysis

The data obtained from this study were analyzed statistically using SPSS software adapted to Windows, version 12. In this software, we used ANOVA and the Student's *t*-test programs, as described in the text.

Generation of transgenic plants expressing bAK and the different forms of AtCGS

Homozygous tobacco plants expressing bAK (received from Gad Galili) (Shaul and Galili, 1992) were crossed with homozygous transgenic tobacco lines expressing F-AtCGS, T-AtCGS (lacking its N-terminus region) or D-AtCGS (lacking 90-nt of the N-terminus region; Hacham *et al.*, 2002, 2006). The expression levels of alien genes were verified by western blot analysis in the leaves of 12-week-old heterozygous F_1 plants. Following self-pollination, 40 F_2 plants from each set were grown in the greenhouse. Four classes of F_2 plants were obtained: carriers of the F-AtCGS, T-AtCGS or D-AtCGS genes; carriers of bAK; carriers of a combination of AtCGS and bAK foreign genes; and plants not expressing foreign genes, which 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. Detection of amino acids was performed by HPLC or GC-MS, as previously described (Hacham *et al.*, 2002; Golan *et al.*, 2005). The detection of soluble methionine and threonine in seeds was performed with 25 mg of dry seeds ground in 1 ml of methanol. After 10 min of centrifugation (4°C, 20 000 ***g***), the supernatants were collected, and 700 μl of chloroform and 375 double distilled water were added to the supernatant. After 30 min of centrifugation at 3000 *g*, 450 μl from the upper water phase was collected, dried and dissolved in 140 μl of 20 mg ml^{-1} methoxyaminhydrochlorid in piridin. For protein-bound amino acid determination, total soluble proteins were extracted from 100 mg of leaves. Leaves were homogenates and were extracted at 4°C in PBS containing 1 mM phenylmethylsulphonylfluoride (PMSF). Following centrifugation (5 min, 16 000 *g*, 4°C), protein concentration was determined using the Bradford method, and a sample of 5 mg of protein was hydrolyzed in 0.3 ml of distilled 6 M HCl at 110°C, for 22 h under vacuum. Samples of the hydrolyzed proteins were analyzed by GC-MS as previously described (Golan *et al.*, 2005).

Measurement of methionine metabolites

The levels of methionine metabolites were detected as previously described. The level of SMM was determined using HPLC (Hacham *et al.*, 2002); LC-MS was used to detect the level of SAM (Hacham *et al.*, 2007); the purge and trap system connected to the GC-MS was used for the measurement of DMS (Hacham *et al.*, 2002).

Immunoblot analysis

Twelve-week-old tobacco leaves were homogenized by mortar and pestle in a buffer containing 100 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM DTT and 1 mM PMSF at 4°C. After 20 min of centrifugation (16 000 *g* at 4°C), the supernatant was collected. Protein samples (10–20 μg) were fractionated on 10% SDS-PAGE and then transferred to a PVDF membrane using the Bio-Rad Protein Trans-Blot apparatus (Bio-Rad, http://www.bio-rad.com). The membrane was blocked overnight at 4°C in 5% (v/v) non-fat dried milk, and was then reacted with anti-hemagglutinin (anti-HA) monoclonal antibodies (Roche, http://www.roche.com) for 2 h at 25°C. Following incubation with mouse anti-IgG antibody conjugated to horseradish-peroxidase under the same conditions, immunodetection was conducted with an enhanced chemiluminescence kit (Pierce, http://www.piercenet.com) according to the manufacturer's instructions.

RNA extraction and northern analysis

Total RNA was extracted from the 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 3-(*N*-morpholino)propanesulphonic acid (MOPS), pH 7.0, and were then transferred onto a nitrocellulose Hybond N membrane (Amersham, http://www.amersham.com). The blots were hybridized for 12 h at 65°C with probes labeled with [α-^{32}P]dCTP (redivue deoxycytidine 5′-[α^{32}P] triphosphate triethylammonium) using the Rediprime kit (Amersham). The probe was a *Sac*I and *Eco*RI fragment of the Arabidopsis CGS. The quantity of 18S rRNAs for 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 (http://www.bioimage.net).

Feeding experiments with Agrobacterium*-mediated transient expression*

Agrobacterium (EHA105 strain) harboring each of the AtCGS forms was grown overnight at 28°C in Luria-Bertani (LB) media supplemented with 25 μg ml^{-1} Kanamycin and 10 μg ml^{-1} rifampicin. A 500-μl volume from the overnight culture was precipitated and resuspended in 10 ml of infiltration media [50 mM 2-(*N*-morpholine)-ethanesulphonic acid (MES), pH 5.6, 0.5% glucose, 1 mM NaH_2PO_4, 0.2 mM acetosyringone, 18.8 mM NH_4Cl, 1.2 mM $MgSO_4{\cdot}7H_2O$, 2 mM KCl, 90 nM $CaCl_2$, 8.9 nM $FeSO_4{\cdot}7H_2O$], and was then left for further growth at 28°C until an OD_{600} of 0.5 was reached. These three types of *Agrobacterium* were used to infiltrate the leaves of *N. betamiana* that were grown in a controlled growth chamber. Forty-eight hours after infiltration, distilled water, 2 mM methionine or 2 mM threonine was injected into the same spot and left for 6 h. The injected discs were cut off from the leaves and frozen in liquid nitrogen until their use for western or northern blot analyses. Five plants were taken for each assay.

In vitro *transcription/translation system*

The cDNA form of AtCGS was amplified as previously described (Hacham *et al.*, 2006). These cDNA forms of AtCGS were ligated into pBlueScript KS^- (Stratagene, http://www.stratagene.com) containing the 3HA epitope tag via *Sac*II and *Sma*I restriction sites, and were then subjected to further analysis. *In vitro* transcription and translation of AtCGS cDNA were performed using the TNT coupled wheat germ system (Promega, Promega). Reaction mixtures (25 μl each) were prepared by utilizing an amino acid mixture supplied by the manufacturer at a final concentration of 4 μM. Final concentrations of 0.5 and 1 mM threonine or methionine were examined. Following incubation at 30°C for 90 min, the reaction was stopped by freezing the samples in liquid nitrogen. AtCGS protein levels were subsequently determined by Western blot analysis using anti-HA monoclonal antibodies.

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