Methionine and threonine synthesis are limited by homoserine availability and not the activity of homoserine kinase in Arabidopsis thaliana

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Summary
Homoserine kinase (HSK) produces O-phospho-L-homoserine (HserP) used by cystathionine γ-synthase (CGS) for Met synthesis and threonine synthase (TS) for Thr synthesis. The effects of overexpressing Arabidopsis thaliana HSK, CGS, and Escherichia coli TS (eTS), each controlled by the 35S promoter, were compared. The results indicate that in Arabidopsis Hser supply is the major factor limiting the synthesis of HserP, Met and Thr. HSK is not limiting and CGS or TS control the partitioning of HserP. HSK overexpression had no effect on the level of soluble HserP, Met or Thr, however, when treated with Hser these plants produced far more HserP than wild type. Met and Thr also accumulated markedly after Hser treatment but the increase was similar in HSK overexpressing and wild-type plants. CGS overexpression was previously shown to increase Met content, but had no effect on Thr. After Hser treatment Met accumulation increased in CGS-overexpressing plants compared with wild type, whereas HserP declined and Thr was unaffected. Arabidopsis responded differentially to eTS expression depending on the level of the enzyme. At the highest eTS level the Thr content was not increased, but the phenotype was negatively affected and the T1 plants died before reproducing. Comparatively low eTS did not affect phenotype or Thr/Met level, however after Hser treatment HserP and Met accumulation were reduced compared with wild type and Thr was increased slightly. At intermediate eTS activity seedling growth was retarded unless Met was supplied and CGS expression was induced, indicating that eTS limited HserP availability for Met synthesis.

Keywords: homoserine, phosphohomoserine, methionine, threonine, homoserine kinase, cystathionine gamma-synthase, threonine synthase.

Introduction
The Asp pathway in plants consists of three branches, one leading to Lys, another to Thr and Ile, and the third to Met (Azevedo et al., 1997; Bryan, 1980) (Figure 1). The branch-point to Lys occurs at 3-aspartic semialdehyde and at O-phospho-L-homoserine (HserP) the pathway branches to Met and to Thr/Ile. The node at HserP is bounded by three enzymes, homoserine kinase (HSK; EC 2.7.1.39), which forms HserP; Thr synthase (TS; EC 4.2.99.2), which forms Thr from HserP; and cystathionine γ-synthase (CGS; EC 4.2.99.9), which converts HserP and Cys into cystathionine, leading in two additional reactions to Met. The HserP node is unique to plants (Giovanelli et al., 1974). In microorganisms the analogous branch-point occurs at Hser, with HserP used specifically for Thr synthesis, and O-succinylhomoserine or O-acetylhomoserine used specifically for Met synthesis (Greene, 1996; Patte, 1996).

A complex interplay of regulations controls metabolic flow through the Asp pathway. Aspartate kinase, the first enzyme, is allosterically inhibited by Lys and Thr. Dihydrodipicolinate synthase, the enzyme at the branch to Lys, is feedback regulated by Lys. Thr feedback inhibits homoserine dehydrogenase, the enzyme at the branch leading to Met, Thr, and Ile. CGS expression is negatively regulated by S-adenosyl-L-methionine (SAM), a metabolite.
derived from Met (Chiba et al., 2003), whereas, TS activity is stimulated by SAM (Curien et al., 1998; Madison and Thompson, 1976).

Numerous studies have documented a dynamic interaction between CGS and TS in controlling the biosynthesis of Met (reviewed in Amir et al., 2002 and Hesse and Hoefgen, 2003). Overexpression of CGS resulted in elevated free Met level, but did not significantly affect Thr level (Chiba et al., 1999; Inaba et al., 1994; Kim et al., 2002). By contrast, when TS activity was repressed Thr level was reduced somewhat, but the Met level was significantly increased (Bartlem et al., 2000; Zeh et al., 2001). An explanation for these observations may come from an examination of the comparative kinetic properties of the competing enzymes (Curien et al., 2003). Arabidopsis CGS, a bi-reactant enzyme, was reported to function in a ping-pong mechanism (Ravanel et al., 1998), meaning that the affinity for one substrate depends on the concentration of the other. Arabidopsis TS is a uni-reactant enzyme and its activity is stimulated by SAM (Curien et al., 1996, 1998). The affinity of CGS and TS for HserP differs by 250-fold, with CGS having the lower affinity. Based on estimates of the physiological concentration of metabolites and the kinetic properties of the enzymes Ravanel et al. (1998) predicted that in Arabidopsis CGS is limited by HserP level, but not the level of Cys; whereas TS is not limited by HserP. If this hypothesis is correct it would suggest that fine scale control of Met synthesis is achieved by stringent control of HserP concentration.

HserP synthesis is catalyzed by HSK as depicted in the following reaction:

$$\text{L-homoserine (Hser)} + \text{MgATP} \rightarrow \text{O-phospho-L-homoserine (HserP)} + \text{MgADP}$$

HSK from plants has not been carefully examined but its potential role in regulation of Met and Thr synthesis has been a point of speculation (Datko et al., 1974). In Arabidopsis HSK appears to be encoded by a single gene located at the At2g17265 locus. The Arabidopsis enzyme produced in recombinant form was recently characterized (Lee and Leustek, 1999). Earlier, the native HSK enzyme from wheat germ was purified to homogeneity and characterized (Riesmeier et al., 1993). The Arabidopsis enzyme was predicted to be plastid localized with a mature molecular weight of 33 kDa. It showed an apparent $K_m$ for L-homoserine of 0.40 mM and for Mg-ATP of 0.32 mM and it required $K^+$ and Mg$^{2+}$ for activity. The wheat germ enzyme was reported to be 36 kDa, showed apparent $K_m$ values of 0.24 mM for L-homoserine and 0.33 mM for Mg-ATP, and it also required $K^+$ and Mg$^{2+}$ for activity. Homoserine kinase is localized exclusively in plastids of pea, a portion of it soluble and another component associated with thylakoid membranes (Muhiitch and Wilson, 1983; Wallsgrove et al., 1983). Evidence for the regulation of HSK is variable. In contrast to earlier studies showing that HSK from pea and radish are allosterically inhibited by Thr, Ile, Val, and SAM (Baum et al., 1983; Thoen et al., 1978) the pure Arabidopsis and wheat germ enzymes were found to be insensitive to these amino acids (Lee and Leustek, 1999; Riesmeier et al., 1993). Greenberg et al. (1988) found that HSK activity was repressed in a methionine-overproducing soybean cell line mutant.

The aim of this study was to investigate the role of HSK in controlling the physiological concentration of HserP and the possible downstream effect on HserP utilization by CGS and TS for Met and Thr biosynthesis.

**Results**

**HSK overexpression in Arabidopsis**

Arabidopsis plants were transformed with a construct for overexpression of HSK under transcriptional control of the CaMV 35S promoter. Thirty-five transgenic plants were isolated. Three plants, 784-5, -17, and -25, that showed a range of HSK expression level were chosen for in-depth analysis and homozygous T3 lines were established from them. Figure 2 shows the results of RNA blot, immunoblot, and homoserine kinase activity measurements. Lines 784-5 and -25 showed the highest level of HSK mRNA and protein whereas line -17 was comparatively lower. Although the level of mRNA and protein was approximately equal in lines 784-5 and 784-25 homoserine kinase activity was threefold lower in line 784-25. The reason for the difference in activity compared with protein level is unknown. In wild-type plants HSK mRNA, protein, and activity were all at the detection limit of the assays used. CGS expression was unaffected by overproduction of HSK (Figure 2b).
membrane association. This result is in contrast to the report of soluble stromal fraction and there was no evidence for the localization of overexpressed HSK protein. Moreover, overexpressed HSK was detected entirely within chloroplasts as predicted from the primary amino acid sequence of the amino terminus, which has the hallmarks of a transit peptide (Lee and Leustek, 1999).

Transported into chloroplasts as predicted from the primary amino acid sequence of the amino terminus, which has the hallmarks of a transit peptide (Lee and Leustek, 1999).

To determine whether overexpressed HSK is incorrectly localized to a subcellular compartment cell fractionation was carried out. Analysis of leaf fractions for HSK activity and targeted to a subcellular compartment cell fractionation was carried out. Analysis of leaf fractions for HSK activity and enzyme activity (c) of whole plant extracts. Plants were grown for 12 days on MS agar medium. The plant line designations are given in the figures. The enzyme activities are the mean of three independent experiments ± standard deviation.

Figure 2. Analysis of transgenic Arabidopsis overexpressing HSK. RNA blot (a), immunoblot (b), and enzyme activity (c) of whole plant extracts. Plants were grown for 12 days on MS agar medium. The plant line designations are given in the figures. The enzyme activities are the mean of three independent experiments ± standard deviation.

Total soluble amino acids were resolved and quantified in leaves of all the primary transgenic plants and in a variety of tissues from the homozygous plants, including roots, young and mature leaves and inflorescences. The HSK transgenic plants showed amino acid profiles that were identical to wild type (data not shown). More specifically, overexpression of catalytically active HSK had no effect on the level of HserP, Met, or Thr. Detailed inspection of the HSK overexpressing plants during their life cycle did not reveal any aberrant phenotypes. The transgenic plants were morphologically identical to the wild-type parental plants.

Overexpressed HSK protein is localized in chloroplasts

To determine whether overexpressed HSK is incorrectly targeted to a subcellular compartment cell fractionation was carried out. Analysis of leaf fractions for HSK activity and HSK protein showed that more than 91% of overexpressed HSK is localized within chloroplasts (not shown). Thus, erroneous localization of overexpressed HSK protein is not an explanation for the lack of effect on amino acid level. The experiment also revealed that HSK has the capacity to be transported into chloroplasts as predicted from the primary amino acid sequence of the amino terminus, which has the hallmarks of a transit peptide (Lee and Leustek, 1999). Moreover, overexpressed HSK was detected entirely within the soluble stromal fraction and there was no evidence for membrane association. This result is in contrast to the report that HSK is partially associated with thylakoids in pea (Muhitch and Wilson, 1983).

Hser treatment of Arabidopsis causes HserP, Met and Thr levels to increase

A hypothesis that would account for the lack of effect of HSK overexpression on downstream metabolites is that Hser availability limits the flux of the pathway to Met and Thr. To test this possibility Arabidopsis seedlings were first grown on Murashige and Skoog (MS)-agar medium for 9 days and then were transferred to freshly prepared medium supplemented with 250 μM Hser for up to 72 h or for 24 h on a range of Hser concentrations up to 250 μM. Analysis of amino acids revealed that HserP, Thr and Met increased markedly in the roots of both wild type and transgenic plants over time and at varying Hser concentrations (Figure 3).

HserP accumulated much more rapidly and to a higher level in the transgenic line than in wild type. After treatment with 250 μM Hser for 6 h, HserP increased 150-fold in the transgenic line compared with 15-fold in the wild type (Figure 3a). The HSK-overexpressing plants also accumulated significantly more HserP when Hser was provided in the growth medium above 50 μM (Figure 3d). The accumulation of HserP after treatment with 250 μM Hser for 6 h was proportional to the level of HSK expression (Figure 4), line 784-5 was the highest, followed by 784-25, and then 784-17. These results indicate that overexpressed HSK is physiologically active and capable of driving HserP accumulation.

Despite their enhanced ability to produce HserP, the HSK-overexpressing plants were not significantly different from wild type with respect to Thr and Met accumulation (Figures 3b,e,c,f, and 4). These amino acids accrued in wild type and HSK-overexpressing transgenics to very high levels after Hser treatment. The maximum level of Thr accumulated after 48 h treatment with 250 μM Hser was 88- to 123-fold greater than in untreated plants and the maximum level of Met was 43- to 81-fold greater.

The dynamics of HserP, Thr, and Met accumulation differed somewhat from each other in wild type and HSK-overexpressing lines. In wild type, HserP and Thr accumulated steadily up to 48 h after exposure to Hser, whereas Met accumulation plateaued after 24 h. In the HSK-overexpressing lines HserP accumulation plateaued after 6 h exposure to Hser. Thr accumulated steadily up to 48 h, whereas Met accumulation plateaued after 24 h, just as it did in wild type. The plateau of HserP in transgenic plants is likely due to the depletion of Hser from the growth medium. The cessation of Met accumulation in the transgenic plants and wild type after 24 h is likely due to the downregulation of CGS activity, resulting from a decline in CGS protein (Figure 5).

The data indicate that Hser availability is a critical limitation for Met and Thr accumulation in Arabidopsis. It is well known that Hser synthesis is controlled by
aspartate kinase and homoserine dehydrogenase. If these control points operate under the experimental conditions defined in Figure 3 one would expect that treatment with Asp would not affect the levels of downstream amino acids. As expected, treatment with Asp up to 250 μM for up to 72 h did not alter the level of soluble HserP, Thr and Met, nor did the treatment affect the levels of Lys or Ile (data not shown). Another indication that Hser treatment specifically affected the content of downstream metabolites is that only the levels of HserP, Thr, Met, and S-methylmethionine (data not presented) were markedly increased after Hser treatment.

HserP, Thr and Met increased only in roots after Hser treatment, not in leaves. The lack of response could be due to an inability of leaves to metabolize Hser or it could be due to an inability of Arabidopsis to transport Hser from roots to leaves. To explore these alternative hypotheses the leaves of 9-day-old 784-17 plants were dissected from the roots and were infiltrated with 250 μM Hser. The results of amino acid analysis are presented in Table 1. The data indicate that direct incubation of leaves with Hser caused HserP, Thr, and Met to increase, supporting the idea that Hser is not easily transported from roots to leaves in Arabidopsis.

CGS overexpression enhances the partitioning of Hser toward Met

The results of metabolite feeding experiments indicated the importance of Hser availability in controlling synthesis of Thr and Met. Superimposed on the regulation of the Asp pathway are specific controls for partitioning of HserP into Thr and Met. Previously CGS overexpression resulted in the overaccumulation of Met in Arabidopsis seedlings (Kim et al., 2002). One of the previously analyzed transgenic lines was used here to examine how CGS overexpression effects metabolite accumulation when Hser is supplied in the incubation medium. The results indicated that CGS overexpression reduced the accumulation of HserP compared with wild type (Figure 6a) and enhanced the accumulation of Met (Figure 6b), but did not have a statistically significant effect on Thr (Figure 6c). The increase in Met after Hser treatment was so large in both wild type and...
367-31 that it dwarfed the 10- to 15-fold increase in Met observed in CGS-overexpressing plants grown without Hser (Kim et al., 2002). In total, the results suggest that when Hser is supplied to CGS-overexpressing plants, Met accumulation is markedly enhanced compared with wild type or CGS-overexpressing plants grown without Hser treatment.

Expression of Escherichia coli threonine synthase in Arabidopsis

Previous work demonstrated that the level of Met was increased and Thr was reduced in Arabidopsis and potato when threonine synthase (TS) expression or activity was reduced (Bartlem et al., 2000; Zeh et al., 2001). Expression of E. coli TS (eTS) increased the Thr level and reduced the Met level in cell cultures of tobacco (Muhitch, 1997). To examine the effect of increased TS activity on the partitioning of Hser into Thr and Met under the experimental conditions used in the current study transgenic Arabidopsis were produced that express eTS. The TS from E. coli differs from Arabidopsis TS in that it does not depend on SAM for activity, therefore, expression of eTS in Arabidopsis would be expected to uncouple Thr synthesis from the level of the end products of Met synthesis. Arabidopsis plants were transformed with a construct for overexpression of eTS under transcriptional control of the CaMV 35S promoter and targeting of the protein to plastids (Muhitch, 1997). Forty-four transgenic plants were isolated that showed a range of eTS expression level. Arabidopsis plants were transformed with a construct for overexpression of eTS under transcriptional control of the CaMV 35S promoter and targeting of the protein to plastids (Muhitch, 1997). Forty-four transgenic plants were isolated that showed a range of eTS expression level. T1 plants with the highest level of eTS mRNA developed severe growth abnormalities that included heavily wrinkled and thickened rosette leaves, flower stalks that were chlorotic, and infertility. These could not be propagated. Other T1 plants with intermediate and low level of eTS expression did not show aberrant phenotypes. These were fertile and could be propagated. Homozygous lines were established from plants 829-2, 829-9, and 829-14, individuals that could be sexually propagated and showed low or intermediate level eTS expression in the screen of T1 generation plants. Q-RTPCR analysis was used to measure eTS mRNA in T3 generation homozygous plants. As anticipated, eTS mRNA was undetectable in wild-type plants, but were measured in the transgenic lines. Plants from line 829-9 showed the highest level of eTS mRNA and 829-2 and 829-14 had significantly less eTS mRNA (Figure 7a). These results were similar to the mRNA blot analysis of the T1 generation parental plants. Consistent with the Q-RTPCR results, enzyme activity measurement revealed that all the transgenic lines had greater TS activity than wild type and line 829-9 had the highest activity (Figure 7b). It was not possible to determine how much greater the physiological TS activity was in the transgenic plants because endogenous Arabidopsis TS depends on the physiological level of SAM. Even so, when measured without and with addition of SAM 829-9 showed 25.2- to 3.8-fold higher TS activity than wild type, 829-2 showed 10.1- and 2.1-fold higher activity and 829-14 showed 6.5- to 1.5-fold higher activity (Figure 7b).

Figure 4. Accumulation of OPH, Met, and Thr after Hser treatment. Samples were treated with 250 μM Hser as described in Figure 3. The data were normalized to the sample with the highest level for a given amino acid, which was assigned a value of 1.0. Each value is a mean calculated from three independent measurements ± standard deviation. The OPH data were derived from plants treated for 6 h and the Met and Thr data were from plants treated for 24 h.

Figure 5. Analysis of CGS expression in Hser-treated Arabidopsis. Root samples from wild-type Arabidopsis treated with 250 μM Hser for various times as described in Figure 3 were immunoblotted with antibody against CGS. Two independent samples were assayed for each time point. The results of immunoblotting are shown at the top of the figure and a photograph of the blotting membrane stained with Coomassie blue before it was reacted with the CGS antibody is shown below to indicate that the samples were equally loaded.
Intermediate level expression of eTS in Arabidopsis causes partial Met auxotrophy and induced CGS expression

Individual plants from the three eTS-expressing lines were morphologically indistinguishable from wild type when they were grown in potting mix. However, when grown on MS agar medium line 829-9 showed stunting of root growth (Figure 8). By contrast, the root morphology of plants from line 829-2 and 829-14 was similar to wild type (only 829-2 is shown in Figure 8). The growth of roots from 829-9 plants was restored when Met was added to the growth medium (Figure 8). Addition of methylthiohydroxybutyrate, a compound that is readily converted to Met in plant cells, or Hser also were able to restore the growth of 829-9 plants (not shown). This result indicates that inhibition may be caused by a failure of 829-9 plants to synthesize enough Met to support root growth.

Interestingly, root stunting in 829-9 occurred only in the early phase of growth. Approximately 20 days after

![Table 1](image)

**Table 1** Effect of Hser treatment on the level of amino acids in isolated leaves

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Asp</th>
<th>HserP</th>
<th>Thr</th>
<th>Met</th>
<th>Ile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>1693.6</td>
<td>± 86.3</td>
<td>10.8</td>
<td>± 11.3</td>
<td>292.1</td>
</tr>
<tr>
<td>Hser-treated</td>
<td>1051.7</td>
<td>± 393.7</td>
<td>4703.6</td>
<td>± 809.8</td>
<td>1744.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>15.4</td>
<td>± 4.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>223.2</td>
<td>± 84.8</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>28.1</td>
<td>± 2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>99.0</td>
<td>± 16.9</td>
</tr>
</tbody>
</table>

Seeds from line 784-17 were germinated and grown for 9 days on MS agar medium. The roots were dissected from the shoots and the shoots incubated in MS liquid medium without or with 250 μM Hser for 9 h under the standard growth conditions. Each data value is the mean of three independent measurements ± standard deviation.

![Figure 6](image)

**Figure 6.** Effect of Hser treatment on the concentration of soluble amino acids in wild type and CGS-overexpressing Arabidopsis. Plants were grown, treated and analyzed as described in Figure 3. Graph and line descriptions are as in Figure 3(a–c) except that the data for CGS-overexpressing plants of line 367-31 (first reported in Kim et al., 2002) are indicated by the circle line symbol.

![Figure 7](image)

**Figure 7.** Analysis of eTS-expressing Arabidopsis. Q-RTPCR data (a) and enzyme activity (b) are shown. The analyses were performed on samples extracted from whole plants grown for 12 days on MS agar medium. The plant line designations for (a) and (b) are given below (b). Q-RTPCR results are given as relative values compared with the mRNA level of ACT2. TS enzyme assay was performed with and without addition of SAM to distinguish between endogenous TS activity and ectopic eTS activity. Each value is the mean of three measurements from independent plant samples ± standard deviation.
germination the roots of these plants began growing even if Met was not supplied. Thereafter, the plants continued to develop into mature and fertile individuals that did not display any other phenotypic aberrations. This observation was made by monitoring the growth of 829-9 plants on MS-agar medium under axenic conditions, discounting the idea that 829-9 plants might possibly begin growth by obtaining Met from microorganisms when cultured in potting mix.

In contradiction to the idea that the 829-9 plants are Met-starved during the early period of growth, analysis of soluble amino acids showed that the levels of Met and SMM were similar to wild type. Indeed, the level of all amino acids in 829-9 plants was identical to wild type when tissue samples from different developmental stages and organs were analyzed. Therefore, to further explore the idea that root stunting in 829-9 is due to an inability to produce Met the expression of CGS was examined. Immunoblot analysis revealed that CGS expression was induced in 10-day old 829-9 plants (Figure 9a) a point in development when root stunting is evident. By comparison, the expression of CGS in 829-2, a line that did not show root stunting, was similar to wild type. In 25-day old 829-9 plants the level of CGS was similar to wild type (not shown) indicating that initiation of root growth correlated with a return of CGS to a wild-type level.

Why is CGS expression induced in young 829-9 plants? Thompson et al. (1982) reported that CGS activity is induced in Lemna minor after treatment with a combination of Thr and Lys. These amino acids in combination are herbicidal because they completely inhibit aspartate kinase causing Met starvation (Green and Phillips, 1974). To examine the conditions that effect CGS expression in Arabidopsis seedlings were treated for 48 or 72 h with Met, a combination of Thr and Lys, or a combination of Thr and Lys and Met (Figure 9b). As previously reported (Chiba et al., 1999) CGS expression was repressed by treatment with Met. Treatment with Thr/Lys induced CGS expression. When plants were treated with Met in combination with Thr/Lys the inducing effect of Thr/Lys was overcome and CGS expression was repressed. By contrast, the expression of serine acetyltransferase (SAT), an enzyme in the cysteine biosynthesis pathway that is not directly involved in Met biosynthesis, was unaffected by the treatments. These results indicate that Met starvation induces CGS expression in Arabidopsis and lends further credibility to the idea that root stunting in 829-9 plants results from Met starvation.

*eTS expression reduces the partitioning of Hser toward Met*

The availability of eTS-expressing Arabidopsis offered the possibility of examining how introduction of an enzyme that competes with CGS for Hser affects the accumulation of Hser-derived metabolites. Line 829-9 could not be
studied in this way because it does not have sufficient root mass for analysis after 9 days growth, however 829-2 and 829-14 were analyzed. Using the same experimental conditions outlined in Figures 3 and 6, eTS-expressing plants were treated with Hser. The results indicated that the accumulation of HserP and Met was markedly reduced in the eTS-expressing plants compared with wild type (Figure 10a,b), whereas Thr accumulation was not significantly affected (Figure 10c). Although the changes in Met and HserP are significant, they are minor when compared in absolute values with the accumulation of Thr.

Discussion

**Hser and HserP limit Met and Thr synthesis**

In this investigation we set out to determine whether HSK plays any role in modifying or controlling the partitioning of Hser toward the metabolic branches to Met and Thr. The results show that HSK overexpression does not increase the HserP concentration when plants are grown autotrophically and has no effect on Met and Thr level. When treated with Hser, however, both HSK-overexpressing and wild-type plants accumulate Met and Thr. The most straightforward explanation for the result is that the level of Hser is itself under strict metabolic control, probably via allosteric regulation of aspartate kinase and homoserine dehydrogenase, and that under physiological conditions HSK, CGS and TS are substrate-limited. These results agree well with the hypothesis put forward by Ravanel et al. (1998) and later elaborated by Curien et al. (2003) that Arabidopsis CGS would normally function well below its maximal rate due to HserP limitation. It is more difficult to predict the physiological dynamics of Arabidopsis TS due to the complexity of the interaction with its substrate HserP and its activator SAM (Curien et al., 1998).

In its fully active state the $K_m[HserP]$ of TS is well below the estimated physiological concentration of HserP, whereas in its basal activity state the $K_m[HserP]$ is above the estimated physiological concentration. Nevertheless, the results in Figure 3 show that Thr synthesis is indeed limited by Hser level.

An insight offered by the HSK-overexpressing model system is that super-accumulation of HserP does not

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![Figure 9](image-url)  
**Figure 9.** Analysis of CGS expression in wild type and eTS-expressing Arabidopsis.  
(a) Wild type, 829-2, and 829-9 Arabidopsis were grown for 10 days on MS-agar medium with plates oriented on edge. Whole plant samples were analyzed by immunoblotting with CGS antibody and with antibody against serine acetyltransferase (SAT) as verification of equal protein loading. Three independent samples were analyzed for each plant line.  
(b) Wild-type Arabidopsis were germinated vertically on MS agar plates. Nine-day-old plants were transferred to medium with the indicated supplement for 2 or 4 days treatment. CGS and SAT were analyzed in whole plant samples by immunoblotting. Two independent samples were assayed for each time point. The concentration of medium supplements was 0.5 mM Met, 1 mM Thr, and 1 mM Lys. Antibodies against CGS and SAT were applied to the immunoblot as a mixture. Neither antibody interferes with immunoreaction of the other as previously reported (Kim et al., 2002).

![Figure 10](image-url)  
**Figure 10.** The effect of Hser treatment on the concentration of soluble amino acids in wild type and eTS-expressing line 829-14 Arabidopsis. Plants were grown, treated and analyzed as described in Figure 3. Graph and line descriptions are also as in Figure 3(a–c) except that the data for eTS line 829-14 are represented by the circle line symbol. The results for 829-2 which are not shown were statistically identical to 829-14.
increase the rate of Met (over the first 24 h) and Thr (over the first 48 h) accumulation after Hser feeding. This result suggests that when Arabidopsis seedlings are provided with 250 μM Hser the rate of TS and CGS are maximal, suggesting that the HserP concentration has saturated these enzymes even at the wild-type level of HSK activity.

Cys availability probably does not limit Met synthesis in Arabidopsis

Two observations indicate that Cys availability does not limit Met synthesis in Arabidopsis. When CGS-overexpressing plants were fed Hser, Met accumulated to far higher levels than in wild type (Figure 6b) indicating that in Arabidopsis there is an excess capacity for Cys production. Secondly, treatment of wild-type Arabidopsis with Cys or glutathione did not enhance Hser-induced Met accumulation nor did it prevent the plateau of Met accumulation observed after 24 h Hser treatment (data not shown). Aubert et al. (1998) came to a different conclusion after they observed that Met did not accumulate after application of Hser to sycamore and Echonochloa cell cultures, whereas HserP and Thr accumulated steadily and to high concentrations. The different response of Arabidopsis, sycamore and Echonochloa to Hser treatment would be interesting to explore because it implies that the metabolites that limit Met biosynthesis may differ depending on the plant species.

Downregulation of CGS after Hser feeding correlates with Met accumulation

HserP and Thr accumulated steadily up to 48 h after Hser treatment (Figure 4a,c). By contrast, Met accumulation ceased abruptly after 24 h (Figure 3b). There are several possible explanations for this phenomenon. They are that a CGS downregulation mechanism was activated, that Cys availability limited further Met synthesis, or that Met was further metabolized. The evidence suggests that the most likely explanation is that a downregulation mechanism was induced. The level of CGS was shown to decline within 24 h after Hser treatment (Figure 5) corresponding precisely with the time that Met accumulation plateaued. CGS expression is downregulated in Arabidopsis by its effector SAM (Chiba et al., 2003). Although SAM is synthesized in the cytoplasm, whereas Met is synthesized in plastids, the level of SAM in plastids is known to be tied to Met in the cell (Ravanel et al., 2004).

Upregulation of CGS expression correlates with Met starvation

A transgenic Arabidopsis plant line expressing eTS (829-9) had an increased level of CGS (Figure 9a). This plant line also showed the highest level of eTS expression among the eTS transgenic lines that were analyzed (Figure 7) and it displayed a delay in root growth that was restored when the plants were supplied with Met (Figure 8). The ability to complement the phenotype indicates that this plant line is unable to produce enough Met to support root growth. Altogether, the results suggest that the increase in steady-state level of CGS is caused by Met starvation. CGS activity was also observed to increase in tobacco cells expressing eTS (Muhitch, 1997). Positive regulation of CGS activity by Met starvation was reported first in Lemma by Thompson et al. (1982). They applied a combination of Thr and Lys, a treatment that was shown to be herbicidal by blocking aspartate kinase and homoserine dehydrogenase (Green and Phillips, 1974) effectively blocking Met synthesis. Combined treatment of Arabidopsis with Thr and Lys also caused a rapid accumulation of CGS (Figure 9b). Moreover, the positive regulation was abolished when the plants were treated with Met at the same time as Thr and Lys. The current result raises the question of whether positive regulation of CGS expression is simply a reversal of the known mechanism for downregulation of CGS (Chiba et al., 1999, 2003), or whether induction of CGS expression by Met-starvation is mediated by an independent system.

Comparison of the Hser treatment data with other published results

The magnitude of the increase in Met and Thr observed after Hser treatment is worth contrasting to other transgenic or genetic models that have been published because the comparison indicates how super-accumulation of Met might be achieved through metabolic engineering. Overexpression of CGS in Arabidopsis was reported to increase Met level by 10- to 15-fold without a major change in Thr content (Inaba et al., 1994; Kim et al., 2002). Reduction of TS activity in Arabidopsis caused Met to accumulate 22-fold but Thr content was severely reduced (Bartlem et al., 2000), and in potato Met content in tubers increased by 30-fold and in leaves by 239-fold, also with a severe reduction in Thr content. By contrast, in the present study, Met accumulated 180-fold above wild type in Hser-treated, CGS-overexpressing plants, and this was accompanied by a significant increase in Thr level. The result indicates that a strategy to increase Met might involve co-expression of CGS along with feedback-insensitive mutant forms of aspartate kinase and homoserine dehydrogenase. Feedback-insensitive mutants have previously been used to deregulate the Asp pathway (Karchi et al., 1993; Shaul and Galili, 1992). It is important to note however, that the present results represent the period of dynamic flux after Hser treatment, not the steady-state condition. It is very likely that the levels of end products could be quite different under steady-state conditions.
Experimental procedures

General methods

Biochemicals were purchased from Sigma (St Louis, MO, USA). Radiochemicals were purchased from New England Nuclear Inc. (Boston, MA, USA). Molecular biology methods were generally as described in Sambrook et al. (1989) with modifications as noted. Immunoblotting and HSK enzyme assays were performed with soluble proteins extracts prepared in 100 mM Tris–HCl, pH 8.5 and cleared of insoluble debris by centrifugation at 14 000 g for 10 min. For TS activity measurement, tissues were extracted with 50 mM KH2PO4, (pH 7.8) and the extracts were dialyzed against the same buffer for 16 h at 4°C. Protein concentration was measured by the Bradford method using BSA as the standard. RNA for blotting and PCR was prepared using Trizol Reagent (Life Technologies Inc., Rockville, MD, USA).

Plant growth conditions

Plants were grown either on potting mix (Promix BX; Premier Horticulture Inc., Rivière-du-Loup, Québec, Canada) watered with one-quarter strength Peters (20:20:20, N:P:K) fertilizer (Grace-Sierra Co., Milpitas, CA, USA) prepared in distilled water, or on agar-solidified nutrient medium containing 0.5x MS nutrients (Life Technologies Inc.), 0.8% (w/v) Bacto Agar, and other chemical supplements as indicated. When plants were treated with amino acids they were first germinated on MS-agar nutrient medium for 9 days with the petri dishes positioned on-edge to direct root growth to the medium surface. The plants were then transferred with sterile forceps to fresh medium with one-quarter strength Peters (20:20:20, N:P:K) fertilizer (Grace-Sierra Co., Milpitas, CA, USA) prepared in distilled water and placed in the dark period. Light intensity was approximately 100 mol m−2 sec−1.

DNA constructions and plant transformation

The full-length HSK cDNA (Lee and Leustek, 1999; GenBank accession number AF082525) was cloned in pFF20 at the SmaI and XbaI sites. An expression cassette consisting of the 35S promoter-HSK cDNA-35S terminator flanked by XbaI for all experiments. The seeds obtained from individ-

Amino acid analysis

Amino acids were measured by HPLC using AccQ-Tag (Waters, Inc. Milford, MA, USA) as described in Kim et al. (2002) with the following modifications. Separation and analysis were performed on 3.9 × 150 mm Waters Nova-Pak C8 column with Nova-Pak C18 guard column using a Waters Corp. 2690 separation module, Millenium32 software version 3.2, and a Waters scanning fluorescence detector with an excitation wavelength of 250 nm and an emission wavelength of 395 nm. Eluent A containing sodium acetate and triethyl-
amine at pH 5.05 was purchased as a premix from Waters Corp. and adjusted pH to 5.8 with 50% (w/v) sodium hydroxide. Eluent B was acetonitrile:water (30:70) and eluent C was acetonitrile:water (60:40). The elution protocol was at a flow rate of 1 ml min−1, 0.5 min 100%; 0.5–1.5 min, linear gradient to 6.2% B; 1.5–32 min gradient to 7.3% B; 32–51 min gradient to 28% B; 51–66 min gradient to 37% B; 66–84 min gradient to 65% B; 84–97 min gradient to 100% B; 97–101 min gradient to 50% C; 101–104 gradient to 100% C; 104–106 min gradient to 100% B; 106–108 gradient to 100% A; and 108–114 min 100% A.

RNA analyses

RNA blotting was carried out on a 1% (w/v) agarose formaldehyde gel and Zeta-probe membrane (Bio-Rad, Inc., Hercules, CA, USA). The HSK probe was prepared by run-off transcription with SP6 RNA polymerase of the HSK cDNA cloned into pZL-1 and linearized with Smal. The run-off reaction contained [α-32P]CTP (3000 Ci mmol−1). The CGS probe was prepared using a random primer labeling kit (Life Technologies Inc.) and [α-32P]dCTP (3000 Ci mmol−1).

Quantitative real-time RT-PCR was performed using an iCycler IQ (Bio-Rad, Inc.). Primers for PCR were eTS-1 (5'-caggcgtttgatgc-3') and eTS-2 (5'-gcgtaccccagctctttcag-3'), ACT2-1 (5'-actgaaga-3') and eTS-3 (5'-acccttcatgcagaa-3') and ACT2-2 (5'-ttgctcataatctctcaga-3'). RNA purified with Trizol Reagent was precipitated with LiCl. First-strand cDNA synthesis was with Superscript III RNase H- Reverse Transcriptase (Invitrogen Inc., Carlsbad, CA, USA). The reaction mixture (20 µl) contained 3 µg of total RNA, 500 ng oligo-dT primer, 0.5 mM each dNTP, 5 mM DTT, 200 U of Superscript III, and Reverse Transcriptase buffer (manufacturer supplied) containing 50 mM Tris–HCl, pH 8.3, 75 mM KCl, and 3 mM MgCl2. RNA was incubated with primer and dNTP for 5 min at 65°C and then chilled on ice for 1 min. Superscript III was added and the reaction incubated for 1 h at 50°C followed by 15 min at 75°C to inactivate Superscript III. The real-time PCR reaction (27 µl) contained 12.5 µl of Platinum PCR SuperMix-UDG (Invitrogen Inc.), 1 pmol of each primer, SYBR Green I (final dilution of 1:125 000), and 2 µl of the RT reaction. The PCR conditions were one cycle for 5 min at 94°C followed by 40 cycles of 30 sec at 94°C, 30 sec at 59°C, and 30 sec at 68°C. Threshold values of individual samples were normalized against Arabidopsis actin2 (ACT2) and the values converted to mRNA copies using standard curves prepared for each target mRNA similar to the method described by Bustin (2000).

Immunoblotting

Twenty micrograms of total protein was blotted after SDS-PAGE onto Immobilon-P membrane (Millipore Inc., Billerica, MA, USA). The membrane was incubated with antibody at a dilution of 1:2000. A horseradish peroxidase-linked antibody and the Renaissance™ chemiluminescence reagent (Perkin-Elmer Life Sciences, Boston, MA, USA) was used to detect immunocomplexes.

HSK antibody was raised in rabbit against recombinant HSK prepared as described in Lee and Leustek (1999). The antibody

1A modified version of pFF19 (Timmermans et al., 1990) in which the Sall site in the polylinker was eliminated and the HindIII site was replaced with Sall.
showed a high degree of non-specific reaction so it was immuno-
purified by reaction of the antibody with antigen blotted after SDS-
PAGE onto Immobilon-P as described by Harlow and Lane (1988).
Other antibodies used in this study include one against CGS (Chiba
et al., 1999) and SAT (Murillo et al., 1995).

Enzyme assays
The HSK assay mixture (100 µl) contained 10 mM MgSO4, 50 mM
KCl, 4 mM L-Hser, 10 mM [γ-32P]ATP (1.54 µCi µmol⁻¹), 100 mM Tris–
HCl (pH 8.5), and 10 µg total protein. The reaction was incubated at
30°C for 10, 20 and 30 min. The reaction was stopped by addition of
20 µl of 20% (w/v) TCA. The samples were chromatographed on PEI-
Cellulose F plates (Merck, Darmstadt, Germany) in methanol, 0.5% (w/v)
NaCl, and ammonia (50:50:1) (Teegarden et al., 1990) at 25°C for 2 h.
The plate was dried and exposed to Kodak X-OMAT film (Eastman Kodak
Company, Rochester, NY, USA). The radioactive
radioactivity measured in a scintillation counter with

Subcellular localization of overexpressed HSK
Protoplasts, chloroplasts, and sub-chloroplast fractions were pre-
pared from transgenic Arabidopsis leaves as described in Rotte and
Leustek (2000). UDP-glucose pyrophosphorylase served as the cy-
otosolic marker and was measured according to Harms et al. (2000).
The chloroplast marker was glyceraldehyde-3-phosphodehydro-
genase and was measured as described by Lunn et al. (1990).

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