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Engineering of cysteine and methionine biosynthesis in potato

Review Article

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Summary. Methionine and cysteine, two amino acids containing reduced sulfur, are not only an important substrate of protein biosynthesis but are also precursors of various other metabolites such as glutathione, phytochelatines, S-adenosylmethionine, ethylene, polyamines, biotin, and are involved as methyl group donor in numerous cellular processes. While methionine is an essential amino acid due to an inability of monogastric animals and human beings to synthesise this metabolite, animals are still able to convert methionine consumed with their diet into cysteine. Thus, a balanced diet containing both amino acids is necessary to provide a nutritionally favourable food or feed source. Because the concentrations of methionine and cysteine are often low in edible plant sources, e.g. potato, considerable efforts in plant breeding and research have been and are still performed to understand the physiological, biochemical, and molecular mechanisms that contribute to their synthesis, transport, and accumulation in plants. During the last decade molecular tools have enabled the isolation of most of the genes involved in cysteine and methionine biosynthesis, and the efficient plant transformation technology has allowed the creation of transgenic plants that are altered in the activity of individual genes. The physiological analysis of these transgenic plants has contributed considerably to our current understanding of how amino acids are synthesised. We focused our analysis on potato (*Solanum tuberosum* cv. Désirée) as this plant provides a clear separation of source and sink tissues and, for applied purposes, already constitutes a crop plant. From the data presented here and in previous work we conclude that threonine synthase and not cystathionine gamma-synthase as expected from studies of *Arabidopsis* constitutes the main regulatory control point of methionine synthesis in potato. This article aims to cover the current knowledge in the area of molecular genetics of sulfur-containing amino acid biosynthesis and will provide new data for methionine biosynthesis in solanaceous plants such as potato.

Keywords: Cysteine biosynthesis – Methionine biosynthesis – Cystathionine gamma-synthase – Cystathionine beta-lyase – Methionine synthase – Serine actetyltransferase – Threonine synthase – Nutritional quality – Potato – Transgenic plants

Abbreviations: *CgS* = Cystathionine gamma-synthase; CbL = Cystathionine beta-lyase; MS = Methionine synthase; SAT = Serine acetyltransferase; TS = Threonine synthase; SAM = S-adenosylmethionine; OPHS = O-phospho-homoserine; GSH = glutathione; OAS-TL = O-acetylserine(thiol)lyase; OAS = O-acetylserine; GC/MS = gas chromatography / mass spectrometry; Met = methionine

Introduction

Molecular plant physiology uses the genetic concept of isolating or creating specific mutants. It allows the targeted generation and selection of biochemical phenotypes. This approach has been used extensively to study photosynthesis, and carbohydrate and fatty acid metabolism (Somerville, 1986; Somerville and Browse, 1991; Gibson et al., 1994). If genes are available expression levels can be directly modified *in vivo*. Relevant targets for such an approach are genes that encode proteins, which by classical biochemical and physiological approaches have been identified as central to the function of a metabolic pathway. Molecularly engineered plants, as compared to classical mutants, do have several advantages: it is possible to obtain a range of effects due to varying expression levels of the expressed gene or the antisense gene, inhibition of enzymes encoded by multigene families can be achieved and tissue- and cell-specific alteration of target enzymes can be obtained. Even if the null mutation is lethal, affected transgenic plants still can be obtained. Therefore, the combination of analysis of several genes of a certain pathway should ultimately allow the dissection of complex regulation mechanisms.

One of the goals of plant genetic engineering has been to create crops that are tailored to provide better nutrition for humans and their domestic animals. Methionine is essentially required in the diets of nonruminant animals and therefore, determines the nutritional value of crops. Major crops, such as corn, soybean, and rice are low in one or more of these amino acids. Currently, these amino acids are supplemented to animal feed to promote optimal growth. Therefore, increasing the content of essential amino acids in crops has achieved a great deal of interest. In plants the amount and thus, the regulation of methionine levels is tightly controlled. A profound knowledge of sulfur metabolism and carbon flow is of high interest to understand this control.

Moreover, the essential amino acids of the aspartate family threonine, lysine, methionine, and isoleucine, are synthesised via a branched pathway with altogether complex regulatory control circuits (Fig. 1) (Bryan, 1980; Giovanelli et al., 1980; Azevedo et al., 1997; Matthews, 1999). Control of synthesis is exerted through feedback inhibition of specific isoforms of aspartate kinase early in the pathway being sensitive to either threonine,

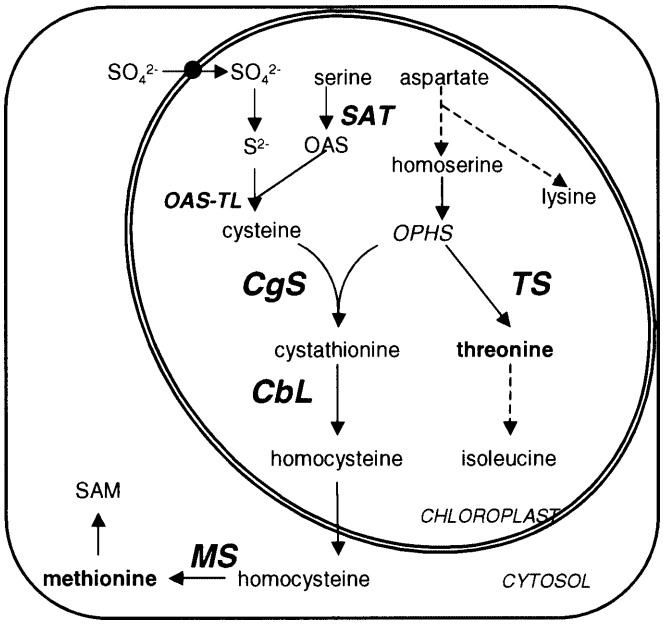

Fig. 1. Biosynthetic pathway of the aspartate amino acid family, sulfur assimilation, and the cysteine biosynthetic pathway in plants. Methionine biosynthesis comprises two biosynthetic domains: first, sulfur assimilation, reduction, and cysteine biosynthesis, and second, one branch of the biosynthetic pathway of the aspartate amino acid family. Dashed lines represent parts of the pathway in which detailed descriptions of the enzymatic steps have been omitted. Most of the pathway is localised in the chloroplast, but the final steps of methionine biosynthesis take place in the cytosol. Methionine serves as a precursor for protein and SAM biosynthesis. *TS* threonine synthase; *CgS* cystathionine gamma-synthase; *CbL* cystathionine beta-lyase; *MS* methionine synthase; *SAM* S-adenosyl methionine

lysine or lysine cooperatively together with S-adenosylmethionine (SAM) (Galili, 1995). In plants the branch point intermediate of threonine and methionine synthesis is O-phospho-homoserine (OPHS), which represents the common substrate for both threonine synthase (TS) and cystathionine gamma-synthase (CgS) (Fig. 1). OPHS is either directly converted to threonine by TS or, in a three step mechanism, to methionine through condensation of cysteine and OPHS to cystathionine, which is subsequently further converted to homocysteine and then methionine by the enzymes CgS, cystathionine beta-lyase (CbL), and methionine synthase (MS), respectively (Anderson, 1990; Hell, 1997; Ravanel et al., 1998; Matthews, 1999). Thus, the close linkage between supply of the carbon backbone and sulfur metabolism is evident and cannot be neglected to understand methionine biosynthesis in higher plants. The use of mutants or transgenic plants altered with respect to the activity of single enzymes have led to improved knowledge about the synthesis of sulfur-containing amino acids (Saito, 2000 and references therein).

In this paper, we will give a brief overview on current knowledge in the area of molecular plant physiology of solanaceous plants and more

specifically, we will provide new information on the role of individual genes involved in cysteine and methionine metabolism in potato plants.

Cysteine biosynthesis

The final step in cysteine synthesis is the incorporation of a reduced sulfur moiety into the β-position of serine. The carbon skeleton is derived from serine via O-acetylserine. The biosynthesis occurs through the enzymes serine acetyltransferase (SAT) and O-acetylserine(thiol)lyase (OAS-TL) (Fig. 1). Most sulfur containing bio-molecules present in plants are synthesised directly or indirectly from cysteine. Consequently, the formation of cysteine is the crucial step for assimilation of reduced sulfur into organic compounds (Giovanelli et al., 1980; Schmidt and Jäger, 1992).

Both enzymes SAT and OAS-TL have been reported to be localised in plastids, mitochondria, and the cytosol of different plant species (Brunold and Rennenberg, 1997; Hell, 1997; Hesse et al., 1999) implying that cysteine synthesis is required in all cellular compartments where protein synthesis does occur (Brunold and Suter, 1982; Rolland et al., 1992; Ruffet et al., 1995).

In both plants and bacteria (Kredich, 1993) SAT forms a complex with OAS-TL suggesting an efficient metabolic channelling from serine to cysteine which prevents the diffusion of the intermediate OAS (Ruffet et al., 1994; Bogdanova and Hell, 1997). Recently, Droux et al. (1998) investigated the SAT/OAS-TL complex *in vitro*. Their results suggest that only the excess of free OAS-TL protein is capable of catalysing cysteine formation. This contradicts the finding that a bi-enzymatic complex is required to channel the intermediate *O*-acetylserine (OAS) (Bogdanova and Hell, 1997).

An important feature of cysteine formation in plants is that the activity of SAT is much lower than the activity of OAS-TL (Nakamura et al., 1987; Ruffet et al., 1994). In support of this observation SAT has been shown to be a low-abundance enzyme in comparison to OAS-TL (Ruffet et al., 1994) and thus, the availability of OAS seems to be rate-limiting for cysteine synthesis *in vitro* (Neuenschwander et al., 1991; Saito et al., 1994a). Furthermore, SAT is feedback-inhibited by cysteine (Saito et al., 1995). As there is considerable evidence that SAT plays an important role in regulating cysteine biosynthesis and sulfate assimilation we decided to directly determine *in vivo* whether the SAT-catalysed reaction is indeed rate-limiting in plant cysteine biosynthesis. To this end, the wild type, cys-sensitive *E. coli* SAT *cysE* gene (Denk and Böck, 1987) was constitutively expressed under control of the cauliflower mosaic virus 35S promoter in transgenic potato plants in order to determine the effect on cysteine and glutathione (GSH) biosynthesis in these plants (Harms et al., 2000).

*Cys*E was targeted to chloroplasts by a translational fusion to the 5′-transit peptide sequence of *rbc*S from *Arabidopsis thaliana*. Besides the high accumulation of the *cys*E mRNA the transgenic plants showed a plastidial localisation of both the *E. coli* SAT protein and enzyme activity as determined

in enriched organelle fractions. Crude leaf extracts of these plants exhibited up to 20-fold higher SAT activity than those prepared from wild type plants. The transgenic potato plants expressing the *E. coli* gene showed not only increased levels of enzyme activity but additionally, exhibited up to twofold higher levels of cysteine and glutathione in leaves compared to control plants. This result demonstrates that under normal conditions, i.e. where sulfur supply is not restricted, the very low endogenous activity of SAT is one of the factors limiting the cysteine biosynthetic pathway, at least in the chloroplast. Interesting is the observation that overexpression of SAT and accumulation of the enzyme in plastids did not overcome a certain threshold which might be due to the cys-sensitivity of the expressed enzyme or by free local cysteine concentrations limiting cysteine synthesis (Noctor et al., 1998; Blaszczyk et al., 1999; Creissen et al., 1999). Feeding experiments with isolated chloroplasts from the transgenic plants with OAS and sulfide resulted in a large increase in cysteine synthesis (Saito et al*.,* 1994b). Thus, OAS and sulfide limit cysteine synthesis. Furthermore, the results demonstrate a direct interaction between the biosynthetic pathways of cysteine and glutathione. The increased levels of cysteine in the transgenic potato plants stimulate the biosynthesis of GSH. Hence, glutathione biosynthesis in potato leaves is limited to some extent by the availability of cysteine. The fact that overexpression of SAT in transgenic potato plants results in increased cysteine and glutathione contents further implies that accumulation of OAS might induce sulfate uptake and reduction which are necessary for cysteine synthesis. These findings support the important role of OAS in the sulfur assimilation pathway. OAS serves not only as substrate for cysteine synthesis, it is also discussed as control metabolite for the pathway itself (Hawkesford, 2000) as supported by recent studies. Expression of sulfur transport proteins, ATP-sulfurylase, and APS reductase are induced by OAS (Neuenschwander et al., 1991; Smith et al., 1995, 1997; Takahashi et al., 1997; Bolchi et al., 1999). However, the thiol content in tubers of transgenic lines was unaffected. Thus, the tuber system seems to be differently regulated than the leaf system. Although SAT is expressed in tubers (data not shown) no significant changes in cysteine and glutathione content could be detected. A reasonable explanation could be that the activation and reduction steps of the sulfate assimilatory pathway are not sufficient to increase the flux towards cysteine formation in tubers. Expression analysis of cytosolic and plastidial ATP-sulfurylase isoforms (Klonus et al., 1994) and isoforms of OAS-TL (H. Hesse, unpublished data) revealed that these genes are less expressed in tubers than in leaves, which might be the cause for the unchanged thiol content. The alterations observed in leaf tissue had no effect on the expression of OAS-TL, the enzyme which converts O-acetylserine, the product of SAT, to cysteine. Only a minor effect on its enzymatic activity was observed. In conclusion, despite the potential interaction and interdependency of SAT and OAS-TL there is no concerted regulation of their respective protein amounts. The manipulation of the internal enzyme activity demonstrates the importance of SAT in plant cysteine biosynthesis and proved that production of cysteine and related sulfur-containing compounds can be enhanced by metabolic engineering of

Table 1. Amino acid composition of plants overexpressing SAT

	WT		26		48	
		± SD		± SD		± SD
methionine	5.2	1.1	4.0	1.2	3.8	0.6
serine	201.8	29.3	106.4	13.2	114.7	20.8
threonine	50.6	17.2	51.7	7.1	56.8	12.5
isoleucine	9.4	2.6	12.1	3.2	10.7	3.2
lysine	7.6	2.3	5.9	2.0	6.1	2.0
aspartate	185.4	76.6	260.9	74.3	266.2	56.7

Free amino acids were extracted from young leaves of *in vitro*-grown plantlets. From each of 5 plants per line 3 samples were pooled and amino acid contents were determined using HPLC separation after OPA derivatisation. The mean value of these measurements (nmoles/g fresh weight) including the standard deviation is presented. The SAT overexpressing lines 26 and 48 are sorted according to the intensity of the mRNA signal in Northern blots: expression is higher in line 48 than in line 26. The transgenic plants did not show any phenotypical changes. *WT* wild type control.

single compounds of the branched biosynthetic system. Yet, tissue dependent differences complicate the system.

The transgenic potato plants expressing the *E. coli* SAT provide a tool to examine whether increased levels of cysteine can lead to enhanced levels of methionine additionally to the observed increases in GSH, given that cysteine is the sulfur donor of methionine (Giovanelli et al., 1980). Therefore, we tested the hypothesis whether it is possible to increase the levels of methionine and further amino acids to improve the nutritional quality of crops. Free amino acids were analysed from leaves of transgenic plants, particularly focussing on the aspartate-derived amino acids (Table 1). With the exception of serine, amino acid levels of the transformants and the control were not significantly altered, especially not in Met content. Serine, the substrate of OAS biosynthesis, was significantly reduced by a factor of two indicating that the increased metabolite flow towards cysteine and the even bigger GSH pool might not be compensated through increased serine biosynthesis resulting in serine reduction. SAM and methionine sulfoxide contents, resulting from the metabolism of methionine and protein bound methionine, were not measured.

Methionine biosynthesis

Methionine is synthesised through three consecutive reactions catalysed by the plastidially localised enzymes cystathionine gamma-synthase (CgS), cystathionine beta-lyase (CbL), and the cytosolically localised enzyme methionine synthase (MS) (Fig. 1). Eventually, about 20% of the methionine is incorporated into proteins while 80% are converted to SAM which comprises the end product of the methionine biosynthetic pathway. Recent studies suggest that methionine synthesis in plants has to be controlled at the

level of competition between CgS and TS for their common substrate OPHS (Bartlem et al., 2000; Zeh et al., 2001a). Nevertheless, it is of importance to analyse also the impact of other enzymes of the biosynthetic pathway on the synthesis of methionine.

Cystathionine beta-lyase

CbL catalyses the second step in higher plant methionine biosynthesis. Transgenic potato plants (*Solanum tuberosum* L. cv. Désirée) were generated expressing a potato CbL (*StCbL*; EC 4.4.1.8) in antisense or sense orientation under control of the cauliflower mosaic virus 35S promoter.

The antisense construct yielded transgenic potato plants with reduced CbL levels (Maimann et al., 2000). Transgenic plants exhibiting leaf CbL activity levels of about 50% below wild type levels were obtained. Higher inhibition rates proved to be lethal. Depending on the remaining CbL activity, we found a decrease in methionine content for the most affected lines, accompanied by increases in cystathionine, cysteine, and homoserine. These remarkable increases probably result from a reduced flow of methionine precursors towards methionine due to the reduced CbL activity in the transgenic plants producing a bottleneck. Levels of aspartate amino acid pathway intermediates (including aspartate, lysine, and threonine) remained essentially unaffected. Unexpectedly, an accumulation of homocysteine, the product of CbL activity, was observed in *StCbL* antisense plants. Explanations for this phenomenon remain speculative. Although it has been described that CgS may catalyse homocysteine formation (Kreft et al., 1994; Ravanel et al., 1995, 1998; Thompson et al., 1982) this assumption is unlikely for two reasons: first, homocysteine formation only takes place in the absence of cysteine, which actually accumulates in the transgenic plants. Second, this direct sulfhydration pathway participates in only 3% of total homocysteine synthesis and has seemingly no physiological significance (Giovanelli et al., 1978; MacNicol et al., 1981). The accumulating homocysteine should be methylated to methionine by MS. However, we observed a decrease in methionine while homocysteine increased. Analysis of MS using Northern- and Western-blots revealed that neither expression level nor protein amount of MS were altered compared to wild type (data not shown). Regulation mechanisms of MS are still not deeply understood. As an alternative explanation one might assume a specific compartmentation of the homocysteine pool, probably in the vacuole, occluding it from access of the cytosolically localised MS. Coinciding with ever reduced CbL activities in the selected *StCbL* antisense lines increasingly devastating phenotypes were observed, demonstrating the indispensable role of CbL for plant growth and development. This conclusion is in agreement with data reported for a methionine requiring *Nicotiana plumbagenifolia* mutant (Negrutiu et al., 1985). Based on feeding various substrates to the mutant plant the authors suggested the mutation to have occurred in the CbL gene. This phenotype can be alleviated upon Met supplementation, suggesting that low Met levels

rather than accumulation of toxic pathway intermediates are responsible for phenotypic effects of CbL transgene expression.

Transgenic potato lines overexpressing CbL show no visible phenotype but reveal an accumulation of both CbL transcript and protein (Maimann et al., 2001). The enzymatic activity of CbL in these lines is up to 2.5-fold higher than that of wild type plants despite the use of the constitutive 35S promoter and an adequate quantity in protein content. GC/MS measurements of aspartate-derived metabolites however, show no significant changes in content of amino acids and pathway intermediates when transgenic and wild type plants are compared. It may be that the increase in total CbL activity is not high enough to enhance the metabolic flow towards Met synthesis. Another, more plausible explanation for the lack of Met accumulation is that cystathionine, the precursor of homocysteine, is not available in sufficient amounts. This assumption is supported by increasing leaf Met levels when cystathionine is supplied via petioles to detached leaves of potato. Because exogenously supplied cystathionine had a stimulating effect on Met biosynthesis in both wild type and transgenic plants overexpressing CbL, it appears that even in wild type the *in vivo* concentration of cystathionine is the limiting factor for CbL activity and methionine biosynthesis. The increase in CbL activity at this step of the pathway does not seem to increase the demand for pathway relevant precursors, which is supported by the fact that there are no changes in expression and protein content of CgS in the transgenic potato plants. CbL overexpression does not change the expression patterns and gene products of other pathway relevant genes as evident from RNA and protein blot analyses. In conclusion, despite the essential role of CbL in plant growth and development, the results demonstrate that homologous overexpression of CbL in *Solanum tuberosum* does not foster an increase in Met synthesis due to the limited cystathionine content in plants. Furthermore, no alterations in metabolite concentrations of the aspartate family pathway or the amino acid cysteine were observed. These results are further supported by the very recent findings of Frankard et al. (2002). We complemented the above mentioned *Nicotiana plumbaginifolia* mutant by targeting the *E. coli* CbL homolog, *met*C, to chloroplasts resulting in full complementation of the mutant phenotype and restoration to wild type like plants. Methionine levels were restored to wild type levels, but no accumulation of Met or derivatives were achieved. Thus, CbL seems to have a low control coefficient for the carbon flow towards methionine synthesis and is further hampered by an insufficient supply of substrate. These observations support the assumption that the availability of OPHS might act as the flux-controlling metabolite in the Met synthesis pathway.

Methionine synthase

The last step of methionine synthesis is localised in the cytosol and catalysed by methionine synthase (MS), which methylates homocysteine to form methionine, using N5-methyltetrahydrofolate as methyl group-donor. The

function of this enzyme is on the one hand the *de novo* synthesis of methionine and on the other hand the regeneration of SAM from S-adenosylhomocysteine after methylation reactions. While bacteria are able to use monoglutameric methyltetrahydrofolate MS from *Catharantus roseus* accepts only the triglutameric isoform. Neither SAM nor cobalamin are required for activity of the cobalamin-independent methionine synthase as demonstrated for MS from *C. roseus* but sucrose or photoassimilates seem to regulate MS gene expression in *C. roseus* and *S. tuberosum* (Eckermann et al., 2000; Zeh et al., 2001b).

A potato cDNA clone, *StMS*1, that encodes a methionine synthase was isolated. MS is a low copy gene as revealed by genomic Southern blots (data not shown). The protein was identified based upon both similarity to methionine synthases from other organisms and the bacterial mutant's ability to synthesise methionine if *StMS*1 is expressed. The low copy gene is differentially expressed in potato organs with elevated levels in flowers, basal levels in sink and source leaves, roots, and stolons, and low levels in stems and tubers. This is in good correlation with protein data except that the protein content in leaves was less than expected from the RNA data. Western blot analysis of subcellular fractions revealed that the protein is located in the cytosol. However, the changing pattern of gene expression during the day/night period implied a light-dependent control of MS-transcription normally seen for enzymes localised in plastids. The expression of MS was shown to be light inducible with its highest expression at midday while during the night expression dropped to a low, basal mRNA level. These RNA data were not confirmed at the protein level since the protein content remained constant over the whole day. Feeding experiments of detached leaves revealed that sucrose or sucrose-derived products are responsible for *StMS*1 induction. This induction can be blocked by treatment with DCMU during the light period. Western analysis revealed that the amount of StMS1 is not affected by either treatment. MS expression is regulated by photoassimilates resulting in a day/night rhythm of RNA abundance which, however, does not detectably alter protein levels of MS.

Methionine is readily converted to SAM, a metabolite with several functions as signal molecule, methyl group donor, or precursor for hormones (ethylene) and hormone-like compounds (e.g. polyamines). The synthesis is catalysed by SAM-synthetase (SAMS). Schröder et al. (1997) identified a correlation between SAMS expression and dehydration stress resulting in an increased expression of SAMS. This finding suggests that under certain conditions plants need increased amounts of Met derivatives. Thus, the hypothesis was tested whether MS, the direct synthesising activity of Met, is as well regulated by dehydration (Fig. 2). Potato plants stressed up to 10 days by drought were analysed on RNA and protein level. Although expression of MS is significantly reduced within 4 days of drought and further reduced till the end of the experiment, the protein content remained constant over the whole experimental period indicating that MS is post-translationally regulated. Furthermore, the reduction of expression of MS is a further evidence for the regulation of MS gene expression by photoassimilates. Drought reduces the

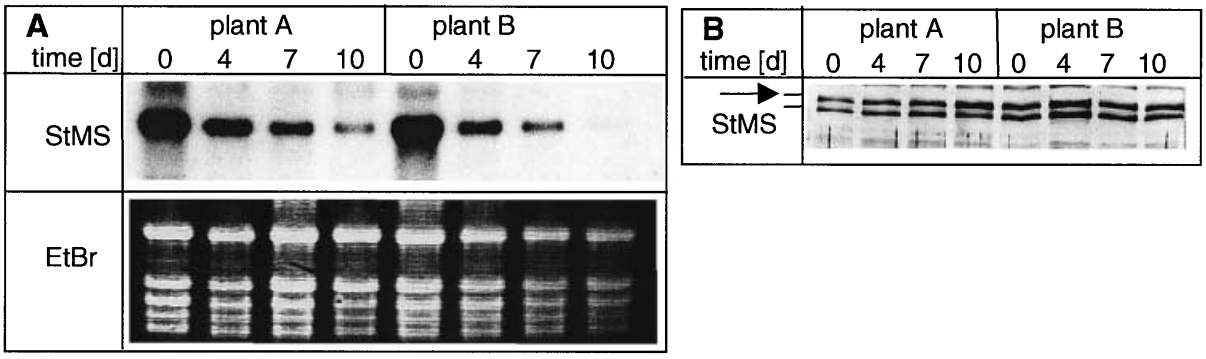

Fig. 2. Time course of expression of methionine synthase during drought stress. Plants kept under greenhouse conditions and cultivated in pots with soil were excluded from watering and samples were taken after 4, 7, and 10 days of drought. **A** RNA blot analysis of total RNA from *S. tuberosum* using *StMS*1 as a probe. Total RNA (40μg) was extracted from leaves, separated by electrophoresis on a formaldehyde gel, blotted onto a nylon membrane, and hybridised to the full size cDNA. Ethidium-bromide stained RNA is shown as a loading control. **B** Western blot analysis of methionine synthase protein with 5μg total protein separated by SDS-PAGE

photosynthetic capacity of plants resulting in less photoassimilates which probably causes the reduced expression. From these data it can be concluded that MS and SAMS expression are not co-regulated by drought stress.

To further investigate the role of MS for the synthesis of Met, homologous expression of MS and antisense approaches have been performed. For both approaches 80 independent transgenic plants of each construct were generated which did not exhibit any phenotypical changes. These were tested on RNA and protein level for changes of expression and protein content, respectively (data not shown). In only few cases a reduced expression of MS was observed but this did not result in changed protein content of the antisense plants. MS overexpressing plants exhibited a moderate increase of MS mRNA signal in Northern blots but this did also not result in changed protein content (data not shown). Analyses of pathway relevant amino acids, such as methionine, threonine, isoleucine, lysine, and aspartate (Table 2), revealed that isoleucine, lysine, and aspartate are not significantly altered. However, methionine contents were marginally though not significantly increased, while threonine levels were increased, though only moderately by a factor of at most 1.8. It can be speculated that overexpression of MS leads to a slight increase of methionine, probably camouflaged by conversion of methionine to SAM. Elevated SAM levels might be made responsible for an increase of threonine through an SAM induced increase of TS activity. This again supports the model presented later and correlates to the models proposed by Giovanelli (1980) and Bryan (1980). On the other hand, we could only achieve a very moderate antisense inhibition. Determination of leaf amino acid contents of methionine, threonine, isoleucine, lysine, and aspartate (Table 3) resulted in somewhat variable and not clearcut results, but

Table 2. Amino acid composition of plants overexpressing MS

line	WT		HH24-20		HH24-116		HH24-48	
		± SD		± SD		± SD		± SD
methionine	2.4	0.2	2.4	0.2	3.2	0.5	3.2	0.9
threonine	62.8	12.0	77.2	7.0	94.8	20.0	113.1	7.3
isoleucine	183.6	16.0	222.6	48.0	231.8	24.2	144.4	36.5
lysine	78.5	24.4	67.1	14.9	62.6	6.3	43.2	8.0
aspartate	172.7	15.4	185.8	15.9	223.4	14.6	215.9	5.5

Free amino acids were extracted from young leaves of *in vitro*-grown plantlets. From each of 5 plants per line 3 samples were pooled and amino acid contents were determined using HPLC separation after OPA derivatisation. The mean value of these measurements (nmoles/g fresh weight) including the standard deviation is presented. The lines 20, 116, 48 of the MS overexpressing plants, termed HH24, are sorted according to the increasing intensity of the mRNA signal in Northern blots. The transgenic plants did not show any phenotypical changes. *WT* wild type control.

Table 3. Amino acid composition of antisense MS plants

line	WT		HH2-82		HH2-56		HH2-78		HH2-71	
		± SD		± SD		± SD		± SD		± SD
methionine	2.4	0.2	7.1	1.1	7.3	0.5	2.7	0.2	2.2	0.2
threonine	62.8	12.0	74.4	10.3	90.0	16.4	89.9	19.3	60.7	21.6
isoleucine	183.6	16.0	172.4	31.4	121.7	17.5	138.5	21.7	167.6	12.7
lysine	78.5	24.4	30.5	3.4	56.5	8.6	48.4	8.3	42.7	7.9
aspartate	172.7	15.4	189.5	14.7	459.0	13.0	302.5	25.2	272.2	37.3

Free amino acids were extracted from young leaves of *in vitro*-grown plantlets. From each of 5 plants per line 3 samples were pooled and amino acid contents were determined using HPLC separation after OPA derivatisation. The mean value of these measurements (nmoles/g fresh weight) including the standard deviation is presented. The lines 82, 56, 78, 71 of the MS antisense plants, termed HH2, are sorted according to the decreasing intensity of the mRNA signal in Northern blots. The transgenic plants did not show any phenotypical changes. *WT* wild type control.

again the observed changes are rather moderate and not significant. This indicates that MS antisense resulted, if at all, in only very minor changes. A determination of metabolites downstream of methionine, such as SAM or S-methylmethionine (Bourgis et al., 1999), has not been performed, but might be helpful to determine whether at least minor changes on the flux of compounds containing reduced sulfur could be achieved. The fact that only moderate alterations of the MS expression and protein levels could be achieved as well through over expression as through antisense inhibition indicates the essential role of MS for methionine biosynthesis but probably even more for the plant one carbon methylation cycle. MS activity is essential for Met synthesis and recycling of SAM and seems to be tightly controlled within narrow borders not allowing greater alterations without affecting plant

growth, thus preventing regeneration of transgenics with grossly altered MS activity.

Threonine synthase

In plants, methionine and threonine biosynthetic pathways diverge at the level of OPHS (cf. Fig. 1). The enzymes CgS and TS compete for the common substrate OPHS with the notable feature that plant TS is activated through SAM, a metabolite derived from methionine. In order to investigate the regulation at this central branch point we engineered potato plants expressing TS in sense and antisense orientation using the constitutive CaMV 35S promoter to drive expression of the transgene. The central position of OPHS in plants is different from other organisms able to synthesise methionine and threonine, such as bacteria and yeasts, in which homoserine, the immediate precursor of OPHS, is the last common substrate (Bryan, 1980; Giovanelli et al., 1980). Threonine and methionine are either incorporated into proteins or serve as precursors for isoleucine or SAM biosynthesis, respectively. SAM is one of the central metabolites in plants involved in methylation reactions and polyamine, ethylene, and biotin biosynthesis (Ravanel et al., 1998). Differences in TS mRNA levels were observed among several different potato plant organs: abundance in flowers, leaves, and roots, and scarcity in stems and tubers (Casazza et al., 2000). Neither precursors (sucrose, oxalacetate, homoserine, OPHS), nor reaction products (phosphate, threonine), nor nitrogenous compounds (glutamine, asparagine) have any effect on expression when fed to detached leaves essentially excluding metabolic regulation of TS transcription (Casazza et al., 2000).

Overexpression of threonine synthase

Whereas fungal and bacterial threonine synthases are not activated by SAM levels (Bryan, 1980), the enzymatic activity of plant TS is activated by low concentrations of SAM, the product of the competing pathway, and is inhibited by cysteine (Thoen et al., 1978; Giovanelli et al., 1984, 1985; Curien et al., 1996). Thus, increasing levels of methionine and hence, SAM induce TS activity. Under these conditions the K_m-values of TS for OPHS have been shown to be 250- to 500-fold lower as compared to the competing enzyme, CgS, favouring carbon flow into threonine biosynthesis in preference to methionine synthesis (Madison and Thompson, 1976, Curien et al., 1996, 1998; Laber et al., 1999). Transgenic tobacco plants expressing a SAM-insensitive *E. coli* TS yielded a five-fold increase of threonine (Muhitch, 1997), whereas the *Arabidopsis thaliana* mutant *mto*2, which displays a reduced TS activity, exhibits a 16-fold decrease in threonine content (Bartlem et al., 2000).

To investigate the role of TS in both threonine and methionine synthesis of potato we overexpressed TS under control of the 35S promoter. Four transgenic lines were selected from 80 plants showing a strong expression of TS (Fig. 3). The increased RNA level in the selected lines #18, #20, #46, and

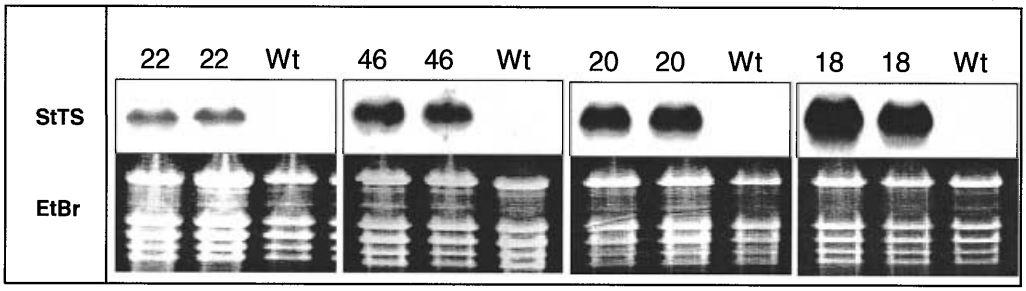

Fig. 3. Overexpression of *StTS* in potato plants. Total RNA (40μg) was extracted from potato leaves, separated by electrophoresis on a formaldehyde gel, blotted onto a nylon membrane, and hybridised to the *StTS* cDNA. Ethidium-bromide stained RNA is shown as a loading control

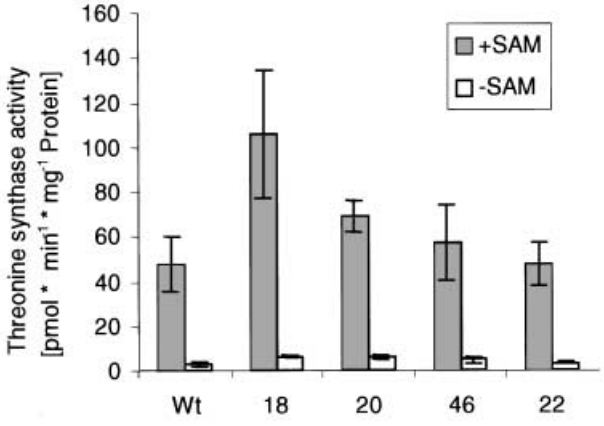

Fig. 4. Threonine synthase activity of TS overexpressing potato plants. For the determination of enzyme activity of TS ^{14}C-labelled OPHS was used as substrate in the presence of the inductor SAM (grey bars) and in its absence (white bars). The data are presented as the mean ± SD of five individual plants per line; three measurements per plant

#22 resulted in an up to 2.5-fold increased enzymatic activity (Fig. 4). Measuring the amino acid content in leaves of lines #18, #20, #46, and #22 revealed no effect on amount and composition of methionine, serine, threonine, isoleucine, lysine, and aspartate compared to wild type (Table 4). Especially, the contents of methionine and threonine were not significantly altered. The newly introduced but homologous enzyme underlies the same regulatory mechanisms as the endogenous enzyme. If activated by SAM TS has a much higher affinity to its substrate OPHS in comparison to CgS, which should enhance the carbon flow to threonine synthesis. However, this is not observed and therefore, it might be assumed that SAM availability controls the carbon flow to threonine. Thus, the surplus of TS cannot be used to promote an increased threonine synthesis as this counteracts methionine and

Table 4. Amino acid composition of plants overexpressing TS

	WT		#20		#22		#46		#18	
		± SD		± SD		± SD		± SD		± SD
methionine	11.4	1.9	9.5	3.9	7.9	1.9	8.5	2.9	9.6	5.7
serine	375.8	152.6	351.5	166.2	319.7	37.0	393.0	204.8	236.3	167.3
threonine	113.6	28.3	107.2	36.6	104.9	25.8	118.6	51.2	78.7	42.5
isoleucine	26.0	3.7	27.3	6.4	26.7	6.2	29.0	6.4	21.5	10.0
lysine	28.2	5.2	25.5	4.1	27.7	6.5	28.4	4.9	31.5	9.1
aspartate	843.7	246.4	669.5	209.7	723.7	217.7	720.9	94.9	578.2	269.7

Free amino acids were extracted from young leaves of *in vitro*-grown plantlets, and amino acid content was determined using HPLC separation after OPA derivatisation. From each of 5 plants 3 samples were pooled and amino acid contents were determined. The mean value of these measurements (nmoles/g fresh weight) including the standard deviation is presented. The TS overexpressing lines 20, 22, 46, 18 are sorted according to the increasing intensity of the mRNA signal in Northern blots. The transgenic plants did not show any phenotypical changes. *WT* wild type control.

consequently SAM accumulation. As soon as SAM levels drop methionine synthesis prevails.

Antisense inhibition of TS

Leaves of TS antisense potato plants exhibit a reduction of TS activity down to 6% of wild type levels. Threonine content is reduced to 45% of wild type controls, whereas methionine levels increase up to 239 fold depending on the transgenic line and environmental conditions. Increased levels of homoserine and homocysteine indicate increased carbon allocation into the aspartate pathway. In contrast to findings in *Arabidopsis thaliana*, where methionine seems to directly control CgS mRNA stability (Inaba et al., 1994; Chiba et al., 1999; Suzuki et al., 2001), increased methionine content has no detectable effect neither on mRNA or protein levels or on the enzymatic activity of CgS in potato. Tubers of TS antisense potato plants contain a methionine level increased by a factor of 30 and no reduction in threonine (Zeh et al., 2001a).

The increased level of Met synthesis in selected transgenic lines correlates with the development of a severe phenotype depending on the amount of accumulated Met (Fig. 5). The leaves start to develop chlorosis (Fig. 5A and C), and the plants are stunted in growth (Fig. 5B). Furthermore, due to a reduced photosynthetic capacity of the transgenic lines the content of soluble sugars, such as glucose, fructose, and sucrose are reduced (Fig. 5D,E, and F).

We tried to alleviate the phenotype through exogenous supplementation of threonine or sulfate in order to test whether the plants are starved for these compounds due to the accumulation of Met on the cost of threonine or internal sulfur stores. Neither spraying nor watering with threonine succeeded in complementing the phenotype (data not shown) even though the same procedure was successfully used to complement CbL antisense plants (Maimann et al., 2000). A possible sulfur deficiency could also be excluded

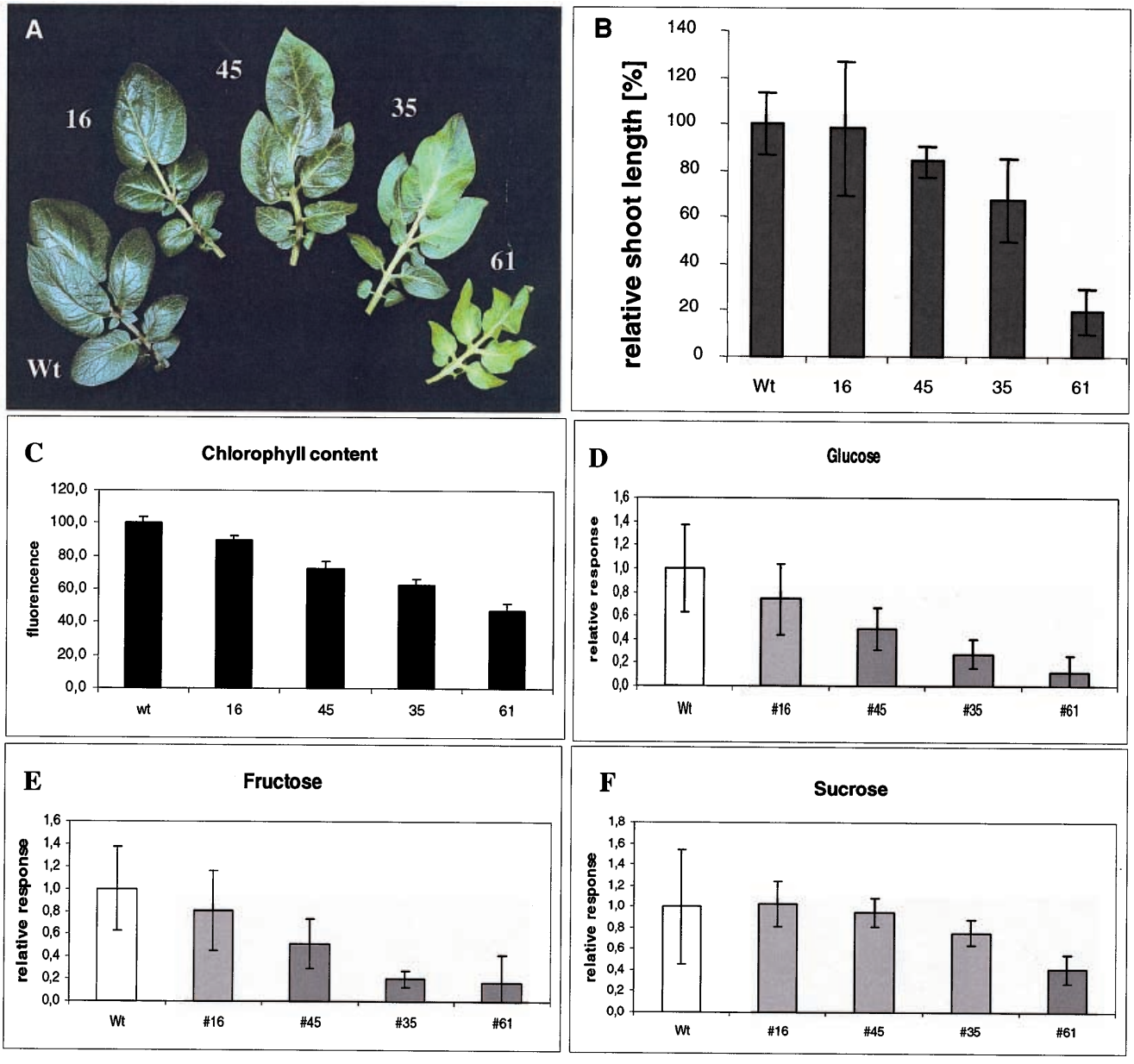

Fig. 5. Phenotype and biochemical characterisation of TS antisense plants reduced in threonine synthase activity. **A** Phenotype of transgenic lines (*16, 45, 35, 61*) and control plants (*Wt*). Line 16 essentially displays wild type appearance whereas lines 45 and 35 display weak symptoms including slight chlorosis along leaf nervature; line 61 shows growth retardation, leaf chlorosis, and alterations in leaf morphology. **B** TS antisense inhibition affects shoot development. **C** Chlorophyll content of leaves from transgenic and wild type plants. **D** Determination of glucose, **E** fructose, and **F** sucrose content of leaves from transgenic and wild type plants. Determination of soluble sugars was performed by GC/MS analysis. Relative responses were determined as area of metabolite to area of internal standard (ribitol). The data are presented as the mean ± SD of five individual plants per line; one measurement per plant

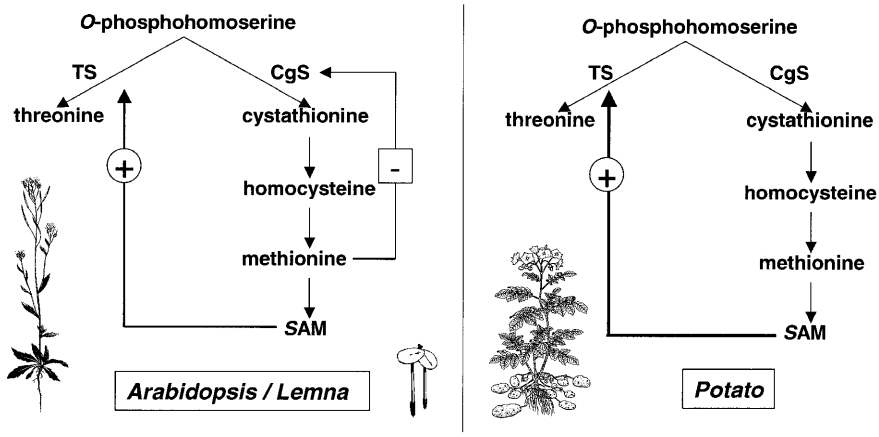

Fig. 6. Comparative model of methionine biosynthesis control of *Arabidopsis thaliana* and *Lemna paucicostata* on the one hand and potato (*Solanum tuberosum*) on the other hand. In *Arabidopsis* and *Lemna* control of methionine biosynthesis occurs through activation of TS activity when SAM accumulates, and a posttranscriptional down-regulation of CgS messenger in case of elevated free methionine concentrations. In potato, however, directing carbon flow either to methionine or threonine biosynthesis seems to be controlled solely by activation of TS enzyme activity in case of SAM accumulation. Methionine, even when increased artificially, does not have any effect on CgS mRNA abundance or enzyme activity. Thus, in potato only the relative enzymatic activities of TS versus CgS control the metabolite flow with first filling the methionine pool and then allocating OPHS into threonine biosynthesis. *TS:* threonine synthase; *CgS:* cystathionine gamma-synthase; *SAM:* S-adenosyl methionine

because fertilising the transgenic plants with additional amounts of sulfate did also not restore the phenotype. Speculatively, the observed phenotype might be caused by accumulation of toxic compounds. Although there is no clear indication that methionine or other intermediates are toxic for the cellular metabolism feeding studies of *Arabidopsis thaliana* with different external concentrations of Met of up to 10 mM revealed that Met in higher concentrations caused first a reduction in shoot and later in root development. Further experiments have to investigate whether Met *per se* or intermediates or derivatives of the pathway are responsible for the observed phenotype. Circumventing these secondary effects is the next step towards generation of crops with improved nutritional value.

Conclusions

Focussing on potato we could unravel a number of regulatory features for methionine biosynthesis. First, though we were able to increase *in vivo* the amount of the precursor cysteine through overexpressing of SAT this did not affect methionine accumulation indicating that not the amount of reduced sulfur (cysteine) is limiting, but rather the availability of the carbon backbone derived from OPHS. The enzymes further downstream, CgS, CbL, and MS, seem not to influence the flow of metabolites even when overexpressed. For

example, CbL was shown to be an essential enzyme as antisense inhibition eventually resulted in lethal phenotypes, and feeding of cystathionine, the immediate substrate of CbL resulted in increased methionine accumulation again indicating the tight control at the level of the carbon backbone. Thus, we investigated the branchpoint between threonine and methionine biosynthesis controlled by CgS and TS. Here, it turned out that a shift of the CgS to TS ratio in favour of CgS resulted in a strong increase of methionine contents. When we reduced TS levels the probably increased levels of SAM following elevated methionine levels were not able to sufficiently activate TS to redirect the carbon flow towards threonine synthesis. In contrast to *Arabidopsis thaliana* we could not observe a downregulation of CgS activity if methionine accumulated in *Solanum tuberosum.* This difference is depicted in a model presented in Fig. 6. For applied purposes it has to be determined whether the targeted crop plant is of the *Arabidopsis* or the potato type concerning the regulation of the CgS / TS branchpoint.

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References

Anderson JW (1990) Sulfur metabolism in plants. In: The biochemistry of plants, vol. 16. Academic Press, New York, pp 327–381

Azevedo RA, Arruda P, Turner WL, Lea PJ (1997) The biosynthesis and metabolism of the aspartate derived amino acids in higher plants. Phytochemistry 46: 395–419

Bartlem D, Lambein I, Okamoto T, Itaya A, Uda Y, Kijima F, Tamaki Y, Nambara E, Naito S (2000) Mutation in the threonine synthase gene results in an over-accumulation of soluble methionine in *Arabidopsis*. Plant Physiol 123: 101–110

Blaszczyk A, Brodzik R, Sirko A (1999) Increased resistance to oxidative stress in transgenic tobacco plants overexpressing bacterial serine acetyltransferase. Plant J 20: 237–243

Bogdanova N, Hell R (1997) Cysteine synthesis in plants: protein-protein interactions of serine acetyltransferase from *Arabidopsis thaliana*. Plant J 11: 251–262

Bolchi A, Petrucco S, Tenca PL, Foroni C, Ottonello S (1999) Coordinate modulation of maize sulfate permease and ATP sulfurylase mRNAs in response to variations in sulfur nutritional status: stereospecific down-regulation by L-cysteine. Plant Mol Biol 39: 527–537

Brunold C, Rennenberg H (1997) Regulation of sulfur metabolism in plants: first molecular approaches. Prog Bot 58: 164–186

Brunold C, Suter M (1982) Intracellular localization of serine acetyltransferase in spinach leaves. Planta 155: 321–327

Bryan JK (1980) Synthesis of the aspartate family and branched-chain amino acids. In: Miflin BJ (ed) The biochemistry of plants, vol 5. Academic Press, New York, pp 403–452

Bourgis F, Roje S, Nuccio ML, Fisher DB, Tarczynski MC, Li CJ, Herschbach C, Rennenberg H, Pimenta MJ, Shen TL, Gage DA, Hanson AD (1999) S-methylmethionine plays a major role in phloem sulfur transport and is synthesized by a novel type of methyltransferase. Plant Cell 11: 1485–1497

Casazza AP, Basner A, Höfgen R, Hesse H (2000) Expression of threonine synthase from *Solanum tuberosum* L. is not metabolically regulated by photosynthesis-related signals or by nitrogenous compounds. Plant Sci 157: 43–50

Chiba Y, Ishikawa M, Kijima F, Tyson RH, Kim J, Yamamoto A, Nambara E, Leustek T, Wallsgrove RM, Naito S (1999) Evidence for autoregulation of cystathionine gamma-synthase mRNA stability in *Arabidopsis*. Science 286: 1371–1374

Curien G, Dumas R, Ravanel S, Douce R (1996) Characterization of an *Arabidopsis thaliana* cDNA encoding an S-adenosylmethionine-sensitive threonine synthase. FEBS Lett 390: 85–90

Curien G, Job D, Douce R, Dumas R (1998) Allosteric activation of *Arabidopsis* threonine synthase by S-adenosylmethionine. Biochem 31: 13212–13221

Creissen G, Firmin J, Fryer M, Kular B, Leyland N, Reynolds H, Pastori G, Wellburn F, Baker N, Wellburn A, Mullineaux P (1999) Elevated glutathione biosynthetic capacity ion the chloroplasts of transgenic tobacco plants paradoxically causes increased oxidative stress. Plant Cell 11: 1277–1291

Denk D, Böck A (1987) L-cysteine biosynthesis in *Escherichia coli*: nucleotide sequence and expression of the serine acetyltransferase (*cysE*) gene from the wildtype and a cysteine-excreting mutant. J Gen Microbiol 133: 515–525

Droux M, Ruffet M-L, Douce R, Job D (1998) Interactions between serine acetyltransferase and O-acetylserine (thiol) lyase in higher plants. Structural and kinetic properties of the free and bound enzymes. Eur J Biochem 255: 235–245

Eckermann C, Eichel J, Schröder J (2000) Plant methionine synthase: New insights into properties and Expression. Biol Chem 381: 695–703

Frankard V, Ispas G, Hesse H, Jacobs M, Höfgen R (2002) Defect in cystathionine β-lyase activity causes the severe phenotype of a *Nicotiana plumbaginifolia* methionine auxotroph. Plant Sci DOI: 10.1016/SO168-9452(01)00603-3

Galili G (1995) Regulation of lysine and threonine synthesis. Plant Cell 7: 899–906

Gibson S, Falcone DL, Browse J, Somerville (1994) Use of transgenic plants and mutants to study the regulation and function of lipid composition. Plant Cell Envir 17: 627–637

Giovanelli J, Mudd SH, Datko AH (1978) Homocysteine biosynthesis in green plants. Physiological importance of the transsulfuration pathway in *Chlorella sorokiniana* growing under steady state conditions with limiting sulfate. J Biol Chem 253: 5665–5677

Giovanelli J, Mudd SH, Datko AH (1980) Sulfur amino acids in plants. In: Miflin BJ (ed) The biochemistry of plants: a comprehensive treatise, vol 5. Academic Press, New York, pp 453–505

Giovanelli J, Veluthambi K, Thompson GA, Mudd SH, Datko AH (1984) Threonine synthase of *Lemna paucicostata* Hegelm. 6746. Plant Physiol. 76: 285–292

Giovanelli J, Mudd SH, Datko AH (1985a) *In-vivo* regulation of *de-novo* methionine biosynthesis in a higher plant *Lemna*. Plant Physiol 77: 450–455

Giovanelli J, Mudd SH, Datko AH (1985b) Quantitative analysis of pathways of methionine metabolism and their regulation in *Lemna*. Plant Physiol 78: 555–560

Harms K, von Ballmoos P, Brunold C, Höfgen R, Hesse H (2000) Expression of a bacterial serine acetyltransferase in transgenic potato plants leads to increased levels of cysteine and glutathione. Plant J 22: 335–343

Hawkesford MJ (2000) Plant responses to sulphur deficiency and the genetic manipulation of sulphate transporters to improve S-utilization efficiency. J Exp Bot 51: 131–138

Hell R (1997) Molecular physiology of plant sulfur metabolism. Planta 202: 138–148

Hesse H, Lipke J, Altmann T, Höfgen R (1999) Molecular cloning and expression analyses of mitochondrial and plastidic isoforms of cysteine synthase (O-acetylserine(thiol)lyase) from *Arabidopsis thaliana*. Amino Acids 16: 113–131

Inaba K, Fujiwara T, Hayashi H, Chino M, Komeda Y, Naito S (1994) Isolation of an *Arabidopsis thaliana* mutant, *mto*1, that overaccumulates soluble methionine. Plant Physiol 104: 881–887

Klonus D, Höfgen R, Willmitzer L, Riesmeier JW (1994) Isolation and characterization of two cDNA clones encoding ATP-sulfurylases from potato by complementation of a yeast mutant. Plant J 8: 105–112

Kreft BD, Townsend A, Pohlenz HD, Laber B (1994) Purification and properties of cystathionine γ-synthase from wheat (*Triticum aestivum* L.). Plant Physiol 104: 1215–1220

Laber B, Maurer W, Hanke C, Gräfe S, Ehlert S, Messerschmidt A, Clausen T (1999) Characterization of recombinant *Arabidopsis thaliana* threonine synthase. Eur J Biochem 263: 212–221

MacNicol PK, Datko AH, Giovanelli J, Mudd SH (1981) Homocysteine biosynthesis in green plants: physiological importance of the transsulfuration pathway in *Lemna paucicostata*. Plant Physiol 68: 619–625

Madison JT, Thompson JF (1976) Threonine synthetase from higher plants: stimulation by S-adenosylmethionine and inhibition by cysteine. Biochem Biophys Res Com 71: 684–691

Maimann S, Wagner C, Kreft O, Zeh M, Willmitzer L, Höfgen R, Hesse H (2000) Transgenic potato plants reveal the indispensable role of cystathionine beta-lyase in plant growth and development. Plant J 23: 747–758

Maimann S, Höfgen R, Hesse H (2001) Enhanced cystathionine β-lyase activity in transgenic potato plants does not force metabolite flow towards methionine. Planta 214: 163–170

Matthews B (1999) Lysine, threonine and methionine biosynthesis. In: Singh BK (ed) Plant amino acids. Marcel Dekker Inc, New York, pp 205–225

Muhitch MJ (1997) Effects of expressing *E. coli* threonine synthase in tobacco (*Nicotiana tabacum*) suspension culture cells on free amino acid levels, aspartate pathway enzyme activities and uptake of aspartate into the cells. J Plant Physiol 150: 16–22

Nakamura K, Hayama A, Masada M, Fukushima K, Tamura G (1987) Measurement of serine acetyltransferase activity in crude plant extracts by a coupled assay system using cysteine synthase. Plant Cell Physiol 28: 885–891

Negrutiu D, De Brouwer D, Dirks R, Jacobs M (1985) Amino acid auxotrophs from protoplast cultures of *Nicotiana plumbagenifolia*, Viviani. Mol Gen Genet 199: 330–337

Neuenschwander U, Suter M, Brunold C (1991) Regulation of sulfate assimilation by light and *O*-acetylserine in *Lemna minor* L. Plant Physiol 97: 253–258

Noctor G, Arisi A-CM, Jouanin L, Kunert KJ, Rennenberg H, Foyer CH (1998) Glutathione: biosynthesis, metabolism and relationship to stress tolerance explored in transformed plants. J Exp Bot 49: 623–647

Ravanel S, Droux M, Douce R (1995) Methionine biosynthesis in higher plants. I. Purification and characterisation of cystathionine γ-synthase from spinach chloroplasts. Arch Biochem Biophys 316: 572–584

Ravanel S, Gakière B, Job D, Douce R (1998) The specific features of methionine biosynthesis and metabolism in plants. Proc Natl Acad Sci USA 95: 7805–7812

Rolland N, Droux M, Douce R (1992) Subcellular distribution of *O*-acetylserine(thiol)-lyase in cauliflower (*Brassica oleracea* L.) inflorescence. Plant Physiol 98: 927–935

Ruffet M-L, Droux M, Douce R (1994) Purification and kinetic properties of serine acetyltransferase free of *O*-acetylserine(thiol)lyase from spinach chloroplasts. Plant Physiol 104: 597–604

Ruffet M-L, Lebrun M, Droux M, Douce R (1995) Subcellular distribution of serine acetyltransferase from *Pisum sativum* and characterization of an *Arabidopsis thaliana* putative cytosolic isoform. Eur J Biochem 227: 500–509

Saito K (2000) Regulation of sulfate transport and synthesis of sulfur-containing amino acids. Curr Opin Plant Biol 3: 188–195

Saito K, Tatsuguchi K, Takagi Y, Murakoshi I (1994a) Isolation and characterization of cDNA that encodes a putative mitochondrion-localizing isoform of cysteine synthase (*O*-acetylserine(thiol)-lyase) from *Spinacia oleracea*. J Biol Chem 269: 28187–28192

Saito K, Kurosawa M, Tatsuguchi K, Tagaki Y, Murakoshi I (1994b) Modulation of cysteine biosynthesis in chloroplasts of transgenic tobacco overexpressing cysteine synthase (*O*-acetylserine(thiol)-lyase). Plant Physiol 106: 887–895

Saito K, Yokoyama H, Noji M, Murakoshi I (1995) Molecular cloning and characterization of a plant serine acetyltransferase playing a regulatory role in cysteine biosynthesis from watermelon. J Biol Chem 270: 16321–16326

Schmidt A, Jäger K (1992) Open questions about sulfur metabolism in plants. Annu Rev Plant Physiol Plant Mol Biol 43: 325–349

Schröder G, Eichel J, Breinig S, Schröder J (1997) Three differentially expressed S-adenosylmethionine synthetases from *Catharanthus roseus*: molecular and functional characterisation. Plant Mol Biol 33: 211–222

Smith FW, Ealing PM, Hawkesford MJ, Clarkson DT (1995) Plant members of a family of sulfate transporters reveal functional subtypes. Proc Natl Acad Sci USA 92: 9373–9377

Smith FW, Hawkesford MJ, Ealing PM, Clarkson DT, Vandenberg PJ, Belcher AR, Warrilow GS (1997) Regulation of expression of a cDNA from barley roots encoding a high affinity sulphate transporter. Plant J 12: 875–884

Somerville C (1986) Analysis of photosynthesis with mutants of higher plants and algae. Annu Rev Plant Physiol 37: 467–507

Somerville C, Browse J (1991) Plant lipids: metabolism, mutants and membranes. Science 252: 80–87

Suzuki A, Shirata Y, Ishida H, Chiba Y, Onouchi H, Naito S (2001) The first exon coding region of cystathionine gamma-synthase gene is necessary and sufficient for downregulation of its own mRNA accumulation in transgenic *Arabidopsis thaliana*. Plant Cell Physiol 42: 1174–1180

Takahashi H, Yamazaki M, Sasakura N, Watanabe A, Leustek T, Engler JA, Engler G, Van Montagu M, Saito K (1997) Regulation of sulfur assimilation in higher plants: a sulfate transporter induced in sulfate-starved roots plays a central role in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 94: 11102–11107

Thoen A, Rognes SE, Aarnes H (1978) Biosynthesis of threonine from homoserine in pea-seedlings. 2. Threonine synthase. Plant Sci Lett 13: 113–119

Thompson GA, Datko AH, Mudd SH, Giovanelli J (1982) Methionine biosynthesis in *Lemna*. Plant Physiol 69: 1077–1083

Zeh M, Casazza AP, Kreft O, Rössner U, Biebrich K, Willmitzer L, Höfgen R, Hesse H (2001a) Antisense inhibition of threonine synthase leads to high methionine content in transgenic potato plants. Plant Physiol 127: 792–802

Zeh M, Leggewie G, Höfgen R, Hesse H (2001b) Cloning and characterisation of a putative cobalamin-independent methionine synthase gene from potato (*Solanum tuberosum* L.). Plant Mol Biol 48: 255–265

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