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Molecular cloning and expression analyses of mitochondrial and plastidic isoforms of cysteine synthase (O-acetylserine(thiol)lyase) from *Arabidopsis thaliana**

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Summary. Cysteine synthase, the key enzyme for fixation of inorganic sulfide, catalyses the formation of cysteine from O-acetylserine and inorganic sulfide. Here we report the cloning of cDNAs encoding cysteine synthase isoforms from *Arabidopsis thaliana*. The isolated cDNA clones encode for a mitochondrial and a plastidic isoform of cysteine synthase (O-acetylserine (thiol)-lyase, EC 4.2.99.8), designated cysteine synthase C (*AtCS-C*, CSase C) and B (*AtCS-B*; CSase B), respectively. *AtCS-C* and *AtCS-B*, having lengths of 1569-bp and 1421-bp, respectively, encode polypeptides of 430 amino acids (~45.8kD) and of 392 amino acids (~41.8kD), respectively. The deduced amino acid sequences of the mitochondrial and plastidic isoforms exhibit high homology even with respect to the presequences. The predicted presequence of AtCS-C has a N-terminal extension of 33 amino acids when compared to the plastidic isoform. Northern blot analysis showed that *AtCS-C* is higher expressed in roots than in leaves whereas the expression of *AtCS-B* is stronger in leaves. Furthermore, gene expression of both genes was enhanced by sulfur limitation which in turn led to an increase in enzyme activity in crude extracts of plants. Expression of the *AtCS-B* gene is regulated by light. The mitochondrial, plastidic and cytosolic (Hesse and Altmann, 1995) isoforms of cysteine synthase of *Arabidopsis* are able to complement a cysteine synthase-deficient mutant of *Escherichia coli* unable to grow on minimal medium without cysteine, indicating synthesis of functional plant proteins in the bacterium. Two lines of evidence proved that *AtCS-C* encodes a mitochondrial form of cysteine synthase; first, import of *in vitro* translation products derived from AtCS-C in isolated intact mitochondria and second, Western blot analy-

*The nucleotide sequence data reported will appear in the EMBL Database under the accession numbers X81973 for *AtCS-C* and X81698 for *AtCS-B*.

sis of mitochondria isolated from transgenic tobacco plants expressing AtCS-C cDNA/c-myc DNA fusion protein.

Keywords: Amino acids – Targeting – Mitochondria – Chloroplasts – Cysteine synthase – Transit peptide – Transgenic plants – Processing

Abbreviations: CSase = cysteine synthase

Introduction

Several important plant processes depend directly or indirectly on the uptake and reduction of sulfate. Reduced sulfur is necessary for the biosynthesis of primary (e.g. amino acids and derivatives, sulfolipids) and secondary (e.g. phytochelatine (Delhaize et al., 1989), sulfated carbohydrates (Schiff, 1973), co-enzyme A, biotin) metabolites (Siegel, 1975; for overview: Schmidt and Jäger, 1992, and references therein). Within this context cysteine biosynthesis represents the essential step in the incorporation of reduced sulfur to organic sulfur in microorganisms and plants. Cysteine synthase (CSase; O-acetyserine (thiol)-lyase, EC 4.2.99.8) catalyses the formation of L-cysteine from O-acetylserine and inorganic and carrier-bound sulfide (Anderson et al., 1990). The enzyme consists of two identical subunits of approximately 35 kD with pyridoxal phosphate as cofactor.

In higher plants the majority of the cysteine synthase activity is located in chloroplasts and cytosol (Brunold and Suter, 1989; Kuske et al., 1996). Lunn et al. (1990) showed that in spinach leaves 15% of the total cysteine synthase activity was located in the mitochondria while the remainder was distributed between the chloroplasts (42%) and cytosol (44%). Cysteine synthase activity in the soluble fraction of disrupted compartments has been shown to correlate with the isolation of cDNAs corresponding to three isoforms of CSase. Recently, cDNAs corresponding to the cytosolic, chloroplastic and mitochondrial isoforms of CSase from spinach (Saito et al., 1992, 1993, 1994), two cytosolic and two chloroplastic isoforms from *Arabidopsis* (Hell et al., 1994; Hesse and Altmann, 1995), one plastidic isoform from pepper (Römer et al., 1992) and one cytosolic isoform from watermelon (Noji et al., 1994) have been described. However, no data relating to the interaction of the various isoforms of the different cell compartments are available. Furthermore, little is known of the molecular genetic organization and regulation of cysteine biosynthesis in plant cells. Although there have been started studies of expression of the three cysteine synthase isoforms (Hell et al., 1994; Takahashi and Saito, 1996), the regulatory mechanisms and responses remained to be analysed. It is therefore important to identify more subcellular isoforms of cysteine synthase to analyse their expression in plant tissues as well as to improve the knowledge of the cysteine biosynthesis for both sulfur assimilation and subcellular interaction.

Analysis of mitochondrial and chloroplastic leader sequences revealed that these signals are extremely heterogeneous in terms of length and sequence composition. There is little structural conservation within either class, although, there is evidence that the information responsible for the

correct targeting of a precursor protein to mitochondria depends on the positive charge and amphiphilicity of the presequence (von Heijne, 1986). In the case of chloroplast transit peptides there is evidence for structurally distinct domains (von Heijne et al., 1989). Both classes of leader sequences show some similarities in their overall amino acid composition, being low in acidic residues and high in hydroxylated amino acids as serine and threonine. In other respects they differ markedly, with the mitochondrial presequence having higher arginine and leucine content and lower threonine content as compared to the chloroplastic transit peptides (Verner and Schatz, 1988; Keegstra, 1989; von Heijne et al., 1989). Despite the low sequence information of possible similarities between these two peptide classes there is little evidence for mistargeting of preproteins. Boutry et al. (1987) found that in tobacco cells transport of a reporter protein to chloroplasts and mitochondria was highly specific depending on the orgin of the leader peptide to which it was fused. Similar results have also been obtained in *in vitro* import experiments (Whelan et al., 1990). Finally, in tobacco cells, a yeast mitochondrial targeting peptide directed transport specifically to mitochondria; no targeting to chloroplasts could be detected (Schmitz and Lonsdale, 1989).

This report describes the cloning and identification of cysteine synthase genes from *Arabidopsis thaliana* involved in the reductive sulfate assimilation pathway in mitochondria and plastids from higher plants in order to get insight into the regulation and compartimentation of sulfate assimilation in different organs and subcellular compartments of higher plants. The presented results suggest that in the case of the mitochondrial isoform of *Arabidopsis* a gene duplication took place and fused a plastidic isoform to a mitochondrial targeting sequence. Whether this combination raises the possibility that under certain environmental conditions this isoform might be shared between these two organelles and plays a biochemical role has to be investigated.

Materials and methods

Strains and plants

Escherichia coli XL1-blue (Stratagene) was cultured using standard techniques (Sambrook et al., 1989). *E. coli* mutant NK3 was kindly provided by Dr. N. M. Kredich (Duke University Medical Center, USA). *Agrobacterium tumefaciens* C58C1 containing the plasmid pGV2260 (Deblaere et al., 1985) was cultured in YEB medium (Vervliet et al., 1975). Tobacco (*Nicotiana tabacum* cv Samsun NN) was propagated in the greenhouse. Tobacco plants in tissue culture were maintained under a 16-hr light/8-hr dark regime on Murashige and Skoog medium (Murashige and Skoog, 1962) containing 2% (w/v) sucrose. Tobacco plants and *Arabidopsis thaliana* in the greenhouse were cultivated in soil. *Arabidopsis thaliana* cv Columbia was propagated in the greenhouse. Plants in the greenhouse were cultivated in soil maintained under a 16-hr light/8-hr dark regime.

To analyse the influence of sulfate, 14 days old *Arabidopsis* seedlings were cultured in tissue culture on Murashige and Skoog medium (1962) containing 1% (w/v) sucrose. Sulfur deprived seedlings were obtained by replacing the sulfate salts in the medium with chloride salts.

cDNA cloning

Approximately 2.5×10^5 Pfu of an *Arabidopsis thaliana* cv Columbia cDNA library derived from mRNA of flowers and very young silics (provided by P. Morris, CNRS, France), constructed in λZAP II XR, was screened under low stringency with a subcloned PCR-amplified genomic fragment encoding cysteine synthase. Primers (Cys I: 5′- GAG AGA GAA TTC GTT GCA AAC ATT GCT GC -3′; Cys II: 5′- GAG AGA CTC GAG TTC AAA ATT TCT TCA GCC TT -3′) used for PCR correspond to homologous regions of published sequences from spinach and pepper (Römer et al., 1992; Saito et al., 1993). Screening was performed at 42°C in a Denhardt buffer accordingly to Sharrock et al. (1988) containing 25% formamide. Filters were washed for 20 min in 3 × SSC, 0.1% SDS at 45°C. Further screening was performed according to the manufacturer's protocol (Stratagene, La Jolla, CA). A full length cDNA for *AtCS-B* was isolated from an *Arabidopsis thaliana* cv Columbia cDNA library derived from mRNA of seedlings (provided by J. Dangle, MPI Köln, Germany), constructed in λgt 10, was screened under high stringency with an incomplete *AtCS-B* cDNA fragment. cDNAs from plaque-purified phage clones were subcloned after digestion with *Eco R*I, agarose-gel purification and ligation into a *Eco R*I predigested pBluescript SK^-.

Nucleic acid manipulations

Both strands of each cDNA insert were sequenced with T7 polymerase (Pharmacia) from a set of subclones (all in pBluescript SK^-) and by using synthetic oligonucleotides. All other methods were performed according to Sambrook et al. (1989).

Total RNA was isolated from greenhouse-grown *A. thaliana* plants according to Logemann et al. (1987). The RNA was fractionated on a 1.2% agarose/formaldehyde gel and blotted to nylon membrane (Hybond N, Amersham). Hybridization was carried out in hybridization buffer according to Sharrock et al. (1988). Washes were performed in 0.2 × SSC, 0.1% SDS at 65°C. The radioactively labeled cDNA inserts of the cytosolic (*AtCS-A*; Hesse and Altmann, 1995), the plastidic (*AtCS-B*) and mitochondrial (*AtCS-C*) isoforms were used as hybridization probes.

Expression in a cysteine-auxotroph, E. coli *NK3*

DNA fragments encoding the mature proteins of cytosolic (*AtCS-A*), plastidic (*AtCS-B*) and mitochondrial (*AtCS-C*) cysteine synthase isoforms were amplified by polymerase chain reaction generating a *Nco*I site at their 5′-ends. Using combinations of oligonucleotides for the cytosolic isoform (AtCSA-N: GAGACCATGGCCTCGA-GAATTGCT/T7 sequencing primer), the plastidic isoform (AtCSB-N: GAGAC-CATGGCTGTATCTATCAAG/T3 sequencing primer) and the mitochondrial form (AtCSC-N: GAGACCATGGCTGTTAAGCGCGAG/T7 sequencing primer) amplified fragments were digested with *Nco*I/XbaI and *Nco*I/Asp718, respectively for the mitochondrial form. Subsequently the cDNAs were directionally cloned in the predigested pKK 388-1 vector (Clontech, USA). Correct amplification of the cDNA fragments were confirmed by DNA sequence analysis. A cysteine auxotroph *E. coli* mutant (*ΔtrpE5 leu-6 thi hsdR hsdM⁺ cysK cysM*) was separately transformed with the generated expression contructs according to Sambrook et al. (1989).

For genetic complementation of the cysteine requirement the transformed *E. coli* was cultured on a M9 minimal agar plate supplemented with 40 μg/ml leucine and tryptophan (Sambrook et al., 1989). The empty cloning vector pKK388-1 was used as a control. IPTG (1 mM) was added to induce expression of proteins from the trc promotor of plasmids pKK388-1 and derivatives.

Determination of cysteine synthase activity

Activity was measured in a volume of 100 μl final volume containing 50 mM KH_2PO_4-K_2HPO_4 (pH 7.5), 5 mM DTT, 10 mM acetylserine and 2 mM Na_2S. After incubation at

25°C for 10 min, the reaction was stopped by addition of 50 µl 20% (w/v) TCA, and the precipitated protein was removed by centrifugation. Cysteine was measured at 560 nm (extinction coefficient of 25,000 $M^{-1} cm^{-1}$) according to the method of Gaitonde (1967).

Mitochondria import assay

Mitochondria were isolated from potato tuber (*Solanum tuberosum* L.) according to Braun et al. (1992). The *AtCS-C* cDNA was expressed by transcription and translation *in vitro*. The mRNA was transcribed with T3 RNA polymerase and subsequently translated in a rabbit reticulocyte extract (Promega) using ^{35}S-Met (800 Ci/mmol) (Amersham). Import of the radiolabeled *AtCS-C* polypeptide into isolated potato mitochondria was carried out as described by Winning et al. (1992). Prior to gel analysis, intact mitochondria were repurified by centrifugation through a 20% (w/v) sucrose cushion.

Construction of two c-myc tagged chimeric genes for AtCS-C

To study the localization of CSase C in plants, two *AtCS-C* cDNA/c-myc gene fusions were constructed by introducing at the 3′-end of *AtCS-C* a nucleotide sequence coding for a c-myc epitope by PCR starting from two different possible start ATGs (+52 and +136). Double-stranded DNAs resulting from three synthetic oligonucleotides (*AtCS*-C-N1: 5′-AGA GAG AGG GTA CCA GGA TCA TGG TGG CGT GAA TGG CTT CAA GG-3′; *AtCS-C*-N2: 5′-AGA GAG TCT AGA GGT ACC TGA ATG GCC GCC ACA TCT TCC TCT GC-3′; *AtCS-C*-C1: 5′-TCT CTC GGA TCC TAC GTA TCA GTT CAG ATC CTC CTC GCT GAT CAG CTT TTG CTC CTC AGG CTG ATC ATT TTC TCC AAC-3′) were ligated to an *Eco R*I- and *Xho* I- predigested pBluescript vector. Both cDNA/c-myc gene fusions (*AtCS-C/c-myc* I and II) were inserted as *Bam HI/Asp* 718 fragments into a binary vector (Becker, 1990) under the contol of the 35S-CaMV promoter. Both plasmids were introduced into tobacco (*Nicotiana tabacum* cv Samsun NN) as described by Rosahl et al. (1987).

Subcellular fractionation and protein analysis

Subcellular fractions of transgenic tobacco plants were obtained as described by Chaumont et al. (1994). The protein concentration was determined by the Bradford (1976) dye-binding assay with BSA as a standard.

Extracted proteins were separated on 12.5% polyacrylamide gels (Laemmli, 1970). For Western blot analysis, proteins were transferred onto nitrocellulose membranes (Schleicher Schüll) using a semi-dry electroblotting apparatus (Multiphor II; LKB Bromma, Sweden). Proteins on membranes were visualized by staining with Ponceau S. Immunodetection was performed using a commercial biotin-streptavidin/alkaline phosphatase system (Amersham) according to the manufacturer's instructions. Antisera raised against the c-myc epitope 9E10 with the peptide AEEQKLISEEDL-LRKRREQLKHKLEQLRNSCA (Dianova, Hamburg, Germany), the potato ADP/ATP translocator (provided by U. Schmitz, University of Hannover, Germany) and the maize fructose 1,6 bisphosphatase (FBPase; provided by B. Buchanan, University of California, Berkeley, USA) were used at a 1:1,000 dilution in TBST-BSA (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Tween-20, 1% BSA).

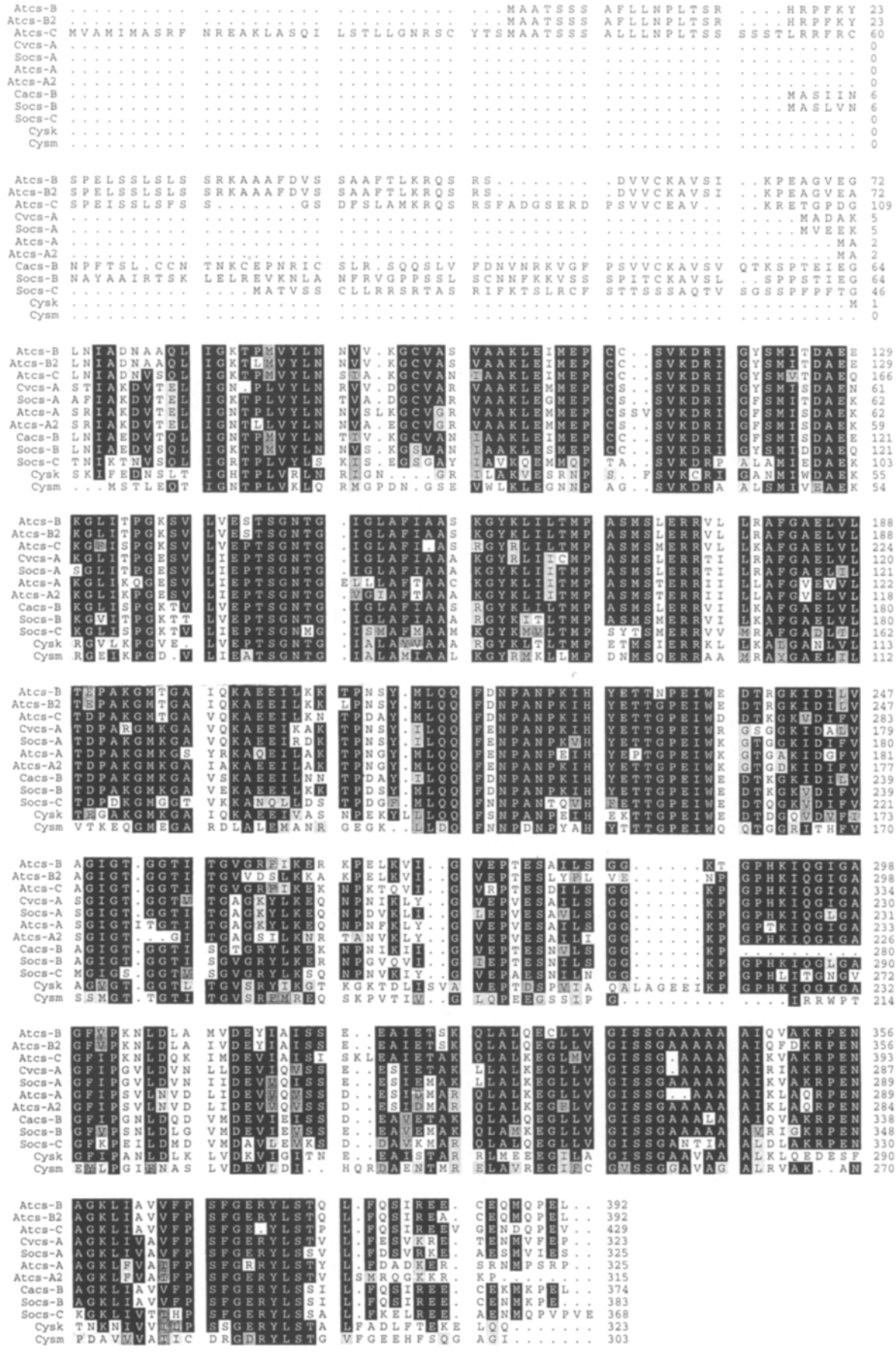
Results

Isolation and sequence analysis of cDNAs encoding cysteine synthase isoforms

A flower specific λZAP II cDNA library was screened using a genomic fragment from *A. thaliana* encoding a conserved region of cysteine synthase

encompassing 300 nt. This fragment cloned in pBluescript SK was obtained by PCR amplification of total *A. thaliana* genomic DNA. The sequence was homologous to sequences of cysteine synthases previously described by Römer et al. (1992) and Saito et al. (1993) and was used to isolate cDNA clones subsequently designated *AtCS-C* and *AtCS-B*. The nucleotide sequence of the PCR amplified fragment was identical to the sequence of *AtCS-C*. The *AtCS-C* cDNA is 1,569 bp in length with an open reading frame of 430-amino acids permitting a protein with a predicted molecular weight of 45.8 kD. A full length cDNA for *AtCS-B* was isolated by screening a seedling λgt10 cDNA library from *Arabidopsis thaliana* with an incomplete *AtCS-B* cDNA. The longest clone was sequenced completely. The *AtCS-B* cDNA is 1,421 bp in length with an open reading frame of 392-amino acids coding for a protein with a predicted molecular weight of 41.8 kD. RNA gel blot analyses of various tissues revealed that the corresponding transcript is approximately 1.5 kb in length, suggesting that the *AtCS-B* cDNA is full length. Sequence comparison of the predicted amino acid sequence with other plant cysteine synthase polypeptide sequences showed that the mature part of all cysteine synthase proteins is highly conserved between different species and compartment specific isoforms (Römer et al., 1992; Saito et al., 1992, 1993, 1994; Hell et al., 1994, Noji et al., 1994; Hesse et al., 1995) and showed similarities between 65 and 90% (Fig. 1). Especially the deduced protein sequences of *AtCS-B* and *-C* have a similarity of 88.7% Homology was also detected between the presequence of mitochondrial cysteine synthase C (*AtCS-C*) and the transit peptide of *AtCS-B*. The major difference detected was a 33 amino acid long N-terminal extension of the putative presequence of *AtCS-C* (this publication; Hell et al., 1994). However, the mitochondrial isoform shows a conserved motif resembling the cleavage site of the transit peptide of chloroplastic isoforms, as determined by protein sequencing for the spinach isoform by Saito et al. (1993) and Rolland et al. (1993). In contrast, the comparison of the putative mitochondrial and the chloroplastic isoforms from spinach are more divergent than the *Arabidopsis* ones (Saito et al., 1993, 1994; Hell et al., 1994). Recently, a cDNA from *Arabidopsis thaliana* encoding a chloroplastic isoform has been published (Hell et al., 1994). The cDNA of *AtCS-B* presented here and the already published cDNA chloroplastic cysteine synthase derived from the same genotype of *Arabidopsis* (cv Columbia) showed a similarity of 92.8%, indicating that chloroplastic cysteine synthase genes are present at least as low copy genes in *A. thaliana*.

Fig. 1. Sequence alignment of the deduced amino acid sequence of *AtCS-C* (accession number of the sequence in this article is X81973) with deduced peptide sequences from *Arabidopsis* (chloroplastic (*AtCS-B*) accession number of the sequence in this article is X81698; cytosolic (AtCS-A), Hesse and Altmann, 1995; chloroplastic (*AtCS-B2*) and cytosolic (*AtCS-A2*), Hell et al., 1994), spinach (mitochondrial (*SoCS-C*), Saito et al., 1994); chloroplastic (*SoCS-B*), Saito et al., 1993; cytosolic (*SoCS-A*), Saito et al., 1992), pepper (plastidic (*CaCS-A*), Römer et al., 1993), watermelon (cytosolic (*CvCS-A*), Noji et al., 1994) and bacteria (cysM and cysK). The predicted amino acids are numbered. The start and stop positions of AtCS-C were used as end points for the alignment



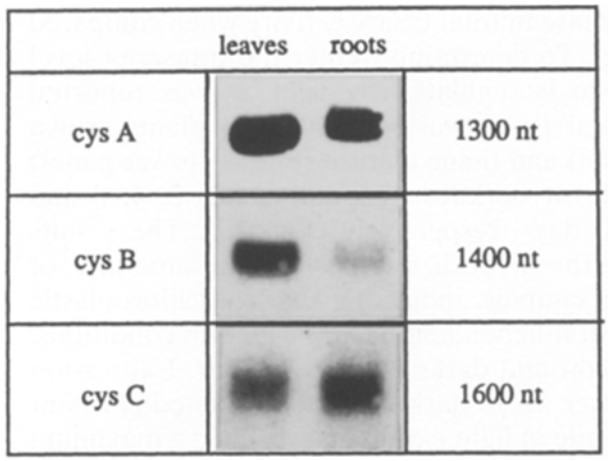
This observation is supported by Southern blot analysis of genomic DNA (data not shown; Hell et al., 1994). Single copy genes were reported in case of the cytosolic isoform in watermelon (Noji et al., 1994) and wheat (Youssefian et al., 1993). In case of spinach, the genomic organization differs (Saito et al., 1992, 1993). Both cytosolic and chloroplastic gene isoforms are encoded by a small gene family. Only the mitochondrial form seems to encode for a single copy gene (Saito et al., 1994).

Expression analysis of cysteine synthase mRNA in A. thaliana

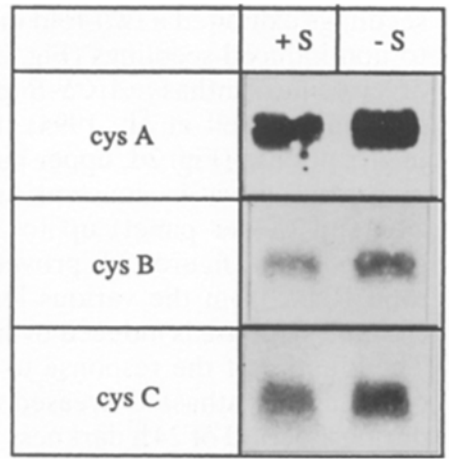
The expression of the cysteine synthase genes in leaves and roots of *A. thaliana* was studied by Northern blot analysis of total RNA samples using *AtCS-A*, *-B* and *-C* cDNAs as probes (Fig. 2a). Despite the high conservation of amino acid sequences, cross-hybridization with transcripts of different isoforms, which were different in transcript size and expression pattern, was not seen. Cysteine synthase transcripts could be detected in each tissue examined, though, at varying levels. In comparison to the expression of the cytosolic isoform *AtCS-A*, which is expressed in similar amounts in roots and leaves (Fig. 2a, *cys A*), the highest amounts of cysteine synthase transcript of *AtCS-B* was observed in leaves (Fig. 2a, *cys* B). The transcript size of 1.5 kb agrees with the length of the isolated cDNA. The *AtCS-C* gene is expressed in both tissues, but higher in roots than in leaves (Fig. 2a, *cys C*). The detected transcript size of 1.6 kb agrees in length with the isolated cDNA.

To determine whether the transcript levels of the cysteine synthase genes are influenced by sulfate, total RNA was isolated from seedlings kept in tissue culture for 14 days in the presence or absence of sulfate in the medium. The result presented in Fig. 2b showed an increase in expression of the mitochondrial cysteine synthase gene (Fig. 2b, *cys C*) as well as the cytosolic (Fig. 2b, *cys A*) and plastidic (Fig. 2b, *cys B*) genes. Corresponding to this measurements of cysteine synthase activity of sulfur starved *Arabidopsis*

Fig. 2. RNA blot analysis of total RNA from *A. thaliana* using *AtCS-A*, *-B*, *-C* as probes. Fifty micrograms of total RNA from **a**) leaves or roots and **b**) sulfur-starved (*−S*) or control (*+S*) seedlings were separated by electrophoresis on a formaldehyde gel, blotted onto a nylon membrane and hybridized to the full size *AtCS-A*, *-B*, *-C* cDNAs. **c** CSase activity in plant crude protein extracts from *Arabidopsis* seedlings. *Arabidopsis* seedlings were starved for 14d or under control conditions. Bar shows S.D. of five different experiments. **d** RNA blot analysis of total RNA from *A. thaliana* using *AtCS-B* as a probe. Fifty micrograms of total RNA were separated by electrophoresis on a formaldehyde gel, blotted onto a nylon membrane and hybridized to the full size *AtCS-B* cDNA. Upper and lower panels: Time course expression of cysteine synthase mRNA during light and darkness. (Upper panel) Total RNA from leaves of greenhouse grown *Arabidopsis* plants; lane 1, control plants; lanes 2–6, grown plants shifted to darkness for 2 h, 4 h, 6 h, 8 h and 24 h, respectively; lanes 7–11, 24-h dark exposed plants returned to light for 2 h, 4 h, 6 h, 8 h and 24 h, respectively. (Lower panel) Expression of cysteine synthase mRNA of 3 weeks old *Arabidopsis* seedlings in tissue culture, lane 1, control; lane 2, 3 and 4 are equivalent to dark exposure times for one, two and three days, respectively


leaves
roots
cys A
cys B
cys C
1300 nt
1400 nt
1600 nt

a


+ S
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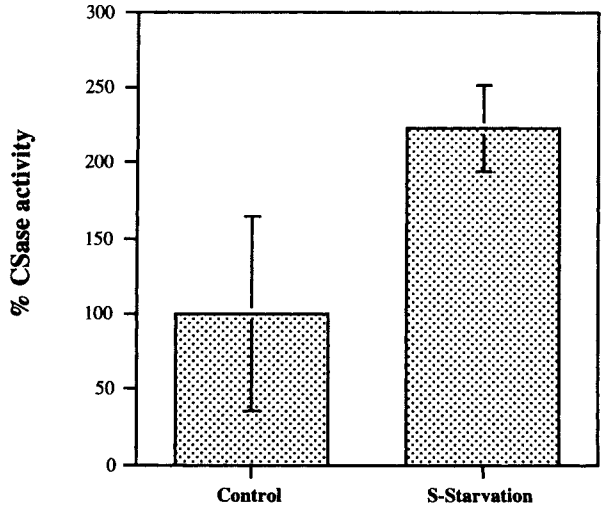
cys C

b


% CSase activity
300
250
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100
50
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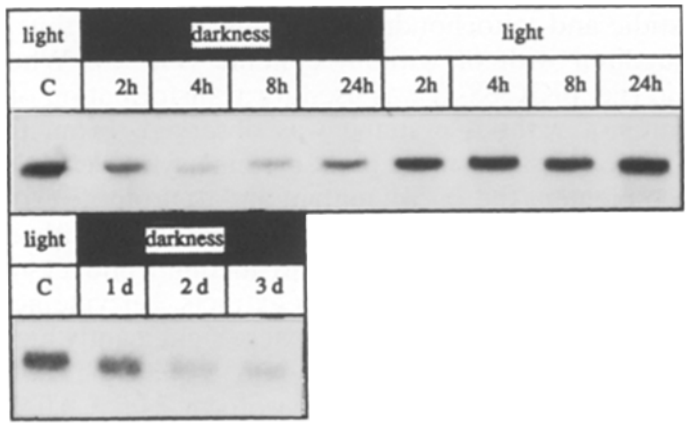
Control
S-Starvation

c


light
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d

seedlings exhibited a two-fold increase in total CSase activity when compared to non-induced seedlings (Fig. 2c). To determine wether the transcript level of cysteine synthase *AtCS-B* gene is regulated by light as was reported previously (Hell et al., 1994), total RNA was isolated from plants grown in greenhouse (Fig. 2d, upper panel) and tissue culture (Fig. 2d, lower panel) at various times in constant light or darkness: (upper panel) 2, 4, 8 and 24 h and (lower panel) up to 3 d days, respectively (Fig. 2d). The results presented in figure 2d proved the hybridization of equal amounts of total RNA from the various leaf samples, indicating that the chloroplastic cysteine synthase is induced by light independent from the growth conditions. The kinetics of the response to light and darkness were similar. Expression of cysteine synthase decreased after 2 h in darkness and remained constant during a period of 24 h darkness while in light expression reached a maximum after 2 h compared to control plants and maintained constant even in a prolonged light period (Fig. 2d, upper panel). Similar results were obtained with plants grown in tissue culture (Fig. 2d, lower panel). Transcript level of cysteine synthase decreased in darkness after one day and remained constant.

Functional expression of recombinant Arabidopsis *cysteine synthase isoforms in* E.coli

We wanted to know whether the isolated *Arabidopsis* cysteine synthase cDNAs did encode for functional proteins. To avoid interferences of the targeting sequences and the mature protein of *AtCS-B* and *-C* only parts of these cysteine synthase cDNAs coding for the mature protein (omitting the presumptive targeting sequence) as well as the entire open reading frame of *AtCS-A* were cloned into the pKK388-1 (Fig. 3). Constructs were then transformed into the cysteine synthase-deficient *E. coli* mutant NK3. *E. coli* mutant cells could be complemented with plasmid constructs of the cytosolic, plastidic and mitochondrial isoforms and were thus able to grow on minimal medium with or without cysteine (Fig. 3). When the empty control plasmid (i.e. pKK388-1) was used for transformation of NK3 growth on cysteine but not without cysteine was observed. From these data we conclude that the expression of the plant cysteine synthase cDNAs is able to functionally complement the *E. coli* mutant and to promote grow on minimal medium. In a second experiment *E. coli* NK3 harboring either *AtCS-A*, *-B*, *-C* or pKK388-1 were cultivated in liquid medium and cysteine synthase activities were estimated in crude protein extracts. Enzyme activities determined for cells expressing the plant genes were significantly higher (cytosolic isoform: 51.2 ± 8.0 nmol mg^{-1} protein min^{-1}; plastidic isoform: 25.4 ± 3.2 nmol mg^{-1} protein min^{-1}; mitochondrial isoform: 24.6 ± 8.0 nmol mg^{-1} protein min^{-1}) than activities present in pKK388-1 cells (3.4 ± 3.0 nmol mg^{-1} protein min^{-1}).

In conclusion, these data clearly indicate the functional expression of *Arabidopsis* cysteine synthases in a prokaryotic background.

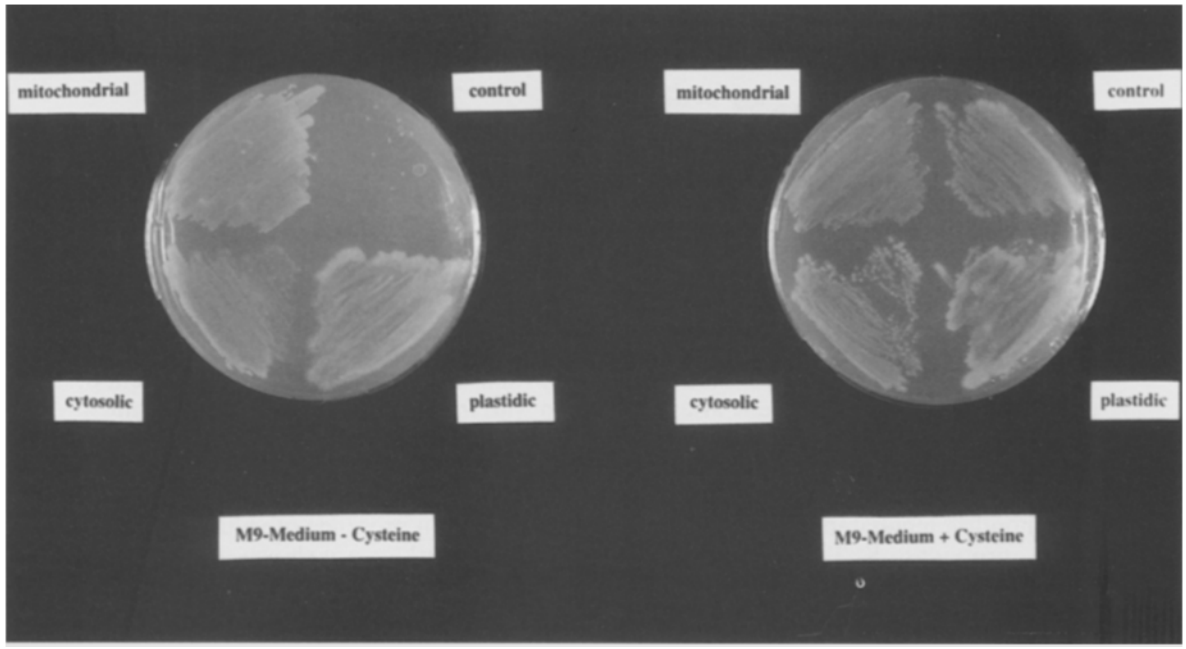

Fig. 3. Complentation of the cysteine synthase- deficient *E. coli* mutant. Plasmids pKK388-1 (empty vector), cytosolic, plastidic and mitochondrial isoforms (harboring the corresponding cDNAs cloned in pKK388-1) were transformed into the *E. coli* mutant NK3 and plated onto M9 minimal medium with or without cysteine (40 μg/ml). Plates were incubated at 37°C

Import of the AtCS-C *polypeptide into mitochondria*

To further support the evidence that *AtCS-C* encodes the mitochondrial isoform of cysteine synthase we analysed the translation product with respect to its ability to be imported into mitochondria by an *in vitro* import assay (Fig. 4). A full-length AtCS-C polypeptide was synthesized *in vitro* with ^{35}S-Met to generate a translation product estimated to be 45 kD in size (Fig. 4, lane 1), which agrees with the calculated size as predicted from the sequence of *AtCS-C*. Smaller translation products in lane 1 may result from either internal initiation or premature termination of translation. After incubation with isolated potato mitochondria, three polypeptides of 45, 44 and 38 kD, also visible in lane 1, and an additional polypeptide of 37 kD were associated with mitochondria (Fig. 4, lane 2). The 37 kD polypeptide was resistant to degradation by proteinase K (Fig. 4, lane 3), indicating that it was localized within mitochondria. When Triton X-100 was added in addition to proteinase K, the 37 kD protein was also degraded (Fig. 4, lane 4). The addition of valinomycin, a potassium ionophore, reduces the import of translation products (Fig. 4, lane 5). These products were associated with the mitochondrial membrane, as indicated by their susceptibility to degradation by proteinase K (Fig. 4, lane 6).

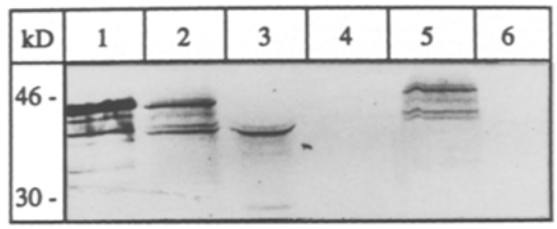

Fig. 4. Import of AtCS-C polypeptide into isolated mitochondria. AtCS-C polypeptide was synthesized *in vitro* in a rabbit reticulocyte extract with ^{35}S-Met. Radioactively labeled protein was analyzed by electrophoresis on a 12.5% (w/v) polyacrylamide gel and autoradiography. Autoradiograms of lane 1: aliquot of *in vitro* translation reaction; lane 2: extract of mitochondria incubated with *in vitro* translation product; lane 3: treatment of mitochondria with proteinase K after incubation with *in vitro* translation product; lane 4: treatment of mitochondria with proteinase K and Triton X-100 after incubation with *in vitro* translation product; lane 5: extract of mitochondria incubated with *in vitro* translation product in presence of valinomycin (1.125 μM final concentration); lane 6: extract of mitochondria incubated with *in vitro* translation product in presence of valinomycin and proteinase K. The positions of molecular mass markers are shown in kD

These results demonstrate that the AtCS-C polypeptide is able to be imported into isolated, intact mitochondria in a membrane potential-dependent manner. It has been demonstrated for some proteins, for example the ADP/ATP translocator, that import requires a membrane potential (Whelan et al., 1988, 1990). Furthermore, the imported AtCS-C polypeptide is smaller than the initial translation product, probably due to the proteolytic cleavage of the presequence after import.

In vivo *localization of the AtCS-C polypeptide*

The presence of an extension of the presequence suggests that the *Arabidopsis AtCS-C* may encode for another organellar isoform of cysteine synthase. We tested whether the *AtCS-C* translation product was able to enter other cell compartments by conducting *in vivo* import assays. Two *AtCS-C*/c-myc gene fusions were constructed and introduced into tobacco plants. Both constructs differ in length with respect to the putative presequence. The *AtCS-C*/c-myc I construct contains the entire open reading frame of *AtCS-C* whereas AtCS-C/c-myc II has a shortened presequence starting at position +33 (Fig. 1) thus deleting the N-terminal extension. Approximately 80 independent transgenic plants of each transformation were screened by Northern and Western blot analysis. For both classes of transgenic plants, transcripts and fusion proteins were detected (data not shown). From these plants a set of three lines per construct were analysed in detail and the results for the line exhibiting the highest amount of expression of the fusion protein were grown in the greenhouse and the results depicted in Fig. 5. To gain insight into the *in vivo* localization of the AtCS-C protein figure 5c shows the result of Western blot experiments obtained for extracts from chloroplasts and mitochondrial fractions of the plants containing either *AtCS-C*/c-myc I or II and of control

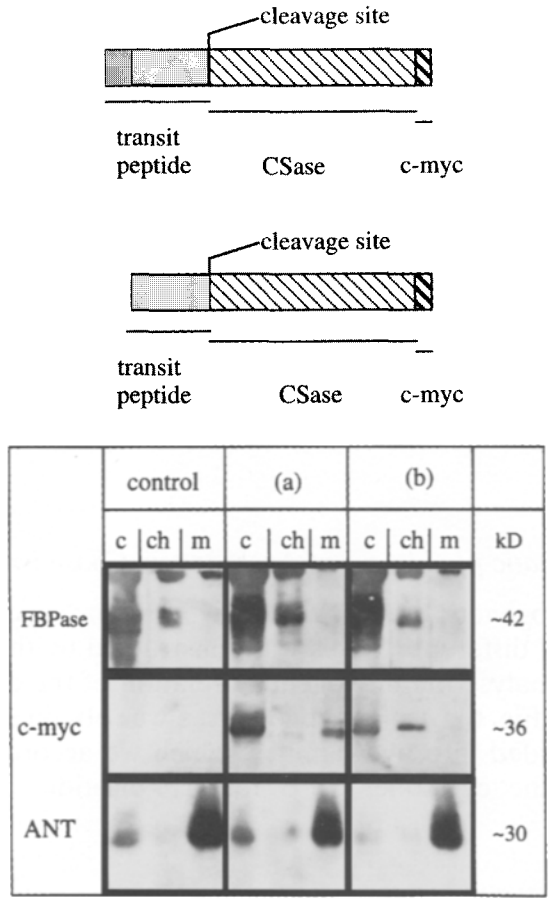

Fig. 5a, b. Schematic representation of the chimeric *AtCS-C*/c-myc fusions. The *AtCS-C* was fused to the c-myc epitope as described in methods. **a** Full-presequence-mature CSase-myc fusion (AtCS-C/c-myc I). **b** Truncated presequence-mature CSase-myc fusion starting at position +33 and the c-myc epitope (AtCS-C/c-myc II). **c** Western blot analysis of subcellular fractions enriched for mitochondria and chloroplasts. Approximately each 30μg of crude extracts (*c*) and each 10μg of protein from chloroplasts (*ch*) and mitochondria (*m*) of transgenic tobacco plants containing either *AtCS-C*/c-myc I or II or the vector only were separated by electrophoresis on a 12.5% (w/v) polyacrylamide gel, blotted onto a nitrocellulose membrane and hybridized to antisera directed against the c-myc epitope (*c-myc*), ADP/ATP translocator (*ANT*) and fructose 1,6 bisphosphatase (*FBPase*)

plants transformed with the "empty" binary vector cassette. The antibody raised against the c-myc epitope detected a fusion protein in crude extracts and enriched mitochondrial fractions in case of plants transgenic for *AtCS-C*/c-myc I and, to a minor extent, in chloroplasts, while in case of plants transgenic for *AtCS-C*/c-myc II the antibody detects a fusion protein in crude extracts and fractions enriched for chloroplasts with the same apparent size that was detected in mitochondria (Fig. 5c, c-myc). The same extracts were tested in Western blots with antisera directed against the mitochondrial ADP/

ATP translocator (Fig. 5c; ANT) and plastidic FBPase (Fig. 5c; FBPase). These results demonstrate that the AtCS-C polypeptide is capable of entering mitochondria only if the complete presequence is present. However, a shortened presequence does not mediate import into mitochondria but rather into chloroplasts. This (mis)targeting into chloroplasts could be due to the fact that the shortened presequence starts with a motif of 16 amino acids which is conserved between the mitochondrial and chloroplastic isoform (Fig. 1). Furthermore, the mitochondrial isoform shows a conserved motif resembling the cleavage site of the transit peptide of chloroplastic isoforms, as determined by protein sequencing for the chloroplastic isoform from spinach by Saito et al. (1993) and Droux et al. (1992). In contrast, the comparison of the mitochondrial and the chloroplastic isoforms from spinach are more divergent than the *Arabidopsis* ones (Saito et al., 1993, 1994).

Enigmatic phylogeny of Arabidopsis *CSase isoforms*

A molecular phylogenetic tree of the derived amino acid sequences of the mature proteins of different CSases was constructed by the maximum likelihood analysis to analyse the phylogenetic relation of the cloned *Arabidopsis thaliana* isoforms (Fig. 6). A phylogenetic tree was obtained where the CSase superfamily is divided into five families, which we according to Saito et al. name CS-A for the cytosolic, CS-B for the plastidic and CS-C for the

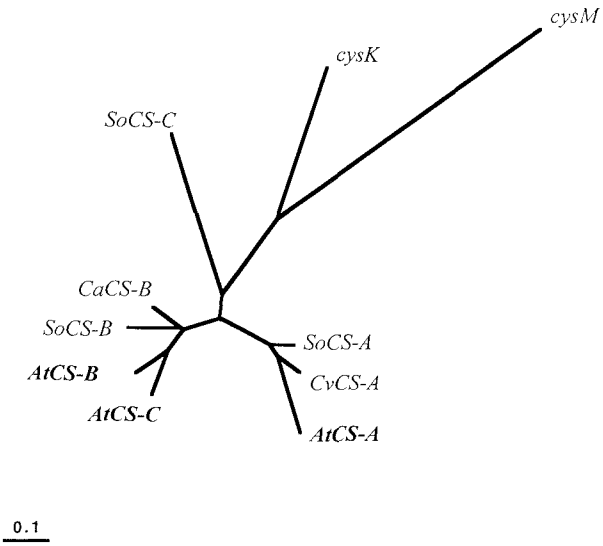

Fig. 6. Molecular phylogenetic tree of CSase proteins. The length of the lines indicate relative distances between nodes. *AtCS-A* cytosolic isoform of *Arabidopsis thaliana*, *AtCS-B* plastidic isoform of *Arabidopsis thaliana*, *AtCS-C* mitochondrial isoform of *Arabidopsis thaliana*, *SoCS-A* cytosolic isoform of *Spinacia oleracea*, *SoCS-B* plastidic isoform of *Spinacia oleracea*, *SoCS-C* mitochondrial isoform of *Spinacia oleracea*, *CvCS-A* cytosolic isoform of *Citrullus vulgaris*, *CaCS-B* plastidic isoform of *Capsicum annuum*, *cysK* and *cysM* bacterial isoforms

mitochondrial form and the bacterial cysM and cysK isoforms. Both the plastidic and mitochondrial *Arabidopsis* isoforms are grouped into the Cys-B cluster, i.e. the plastidic isoforms of CSase though we have shown functionally the plastidic localization of AtCS-B (data not shown) and the mitochondrial localization of *AtCS-C*, which seems to contradict the current model of the evolutionary relationship of the different CSase isoforms. However, the different localization of the two isoforms has been shown by *in vitro* and *in vivo* uptake experiments to depend exclusively on the specific structure of the N-terminal sequences where a mitochondrial targeting sequence seems to be added 5′ to a conventional transit peptide to form a new N-terminal sequence functional as mitochondrial targeting sequence. We therefore speculate that probably based on the duplication of a nuclear gene carrying a plastid transit peptide one of the copies has acquired an additional mitochondrial targeting sequence, thus, redirecting an isoform originally localized in plastids to mitochondria.

Discussion

Two cysteine synthase cDNA clones, designated *AtCS-C* and *-B*, were isolated from a flower and a seedling cDNA library, respectively, by screening with a PCR amplified genomic fragment from *A. thaliana*. Primers were generated to homologous regions from published sequences of spinach (Saito et al., 1993) and pepper (Römer et al., 1992). From the structural features of *AtCS-C* and *-B* cDNAs from *A. thaliana* it was not possible to deduce the subcellular localization of the protein. The predicted open reading frames encode proteins of 45 kD and 41.8 kD, respectively. Interestingly, both the mitochondrial and chloroplastic isoforms show two conserved motifs: the first 16 amino acid residues of the plastidic isoform and a stretch of amino acids resembling the processing site deduced from N-terminal amino acid sequencing of isolated protein from spinach is conserved in composition between different isoforms presumably localized in plastids (Rolland et al., 1993; Saito et al., 1993). Furthermore, AtCS-C comprises a novel extension of 33 amino acids at the N-terminus (Fig. 1), supporting the assumption that the *AtCS-C* product is localized in a subcellular compartment and not in the cytosol. The deduced sequence of the mature protein of *AtCS-C* shows high conservation to other known sequences (cytosolic, chloroplastic and mitochondrial isoforms of spinach: 82.9, 83.8% and 71.1%, respectively; cytosolic and chloroplastic isoforms of *Arabidopsis*: 79.5 and 88.7% (Fig. 2), respectively; pepper: 85.5% and watermelon: 83.5%). AtCS-B shows 92.4% similarity to a plastidic isoform recently published, which was isolated from the same ecotype (Hell et al., 1994).

The Southern blot analysis of cysteine synthase genes suggested a low copy number under stringent hybridization (50% formamide) and washing conditions (0.2 × SSC, 0.1% SDS, 65°C) in the genome of *A. thaliana* (data not shown). Single copy genes were reported in case of the cytosolic isoform in watermelon (Noji et al., 1994), wheat (Youssefian et al., 1993) and the

mitochondrial isoform in spinach (Saito et al., 1994). In case of spinach, the genomic organization differs (Saito et al., 1992, 1993). Both cytosolic and chloroplastic gene isoforms are encoded by a small gene family. RNA blot analysis reveals a different expression pattern for cysteine synthase isoforms. Each probe hybridized only with one transcript differing in length (*AtCS-A* about 1.3 bp; *AtCS-B* about 1.5 bp; *AtCS-C* about 1.6 bp) and amount. While the cytosolic isoform is constitutively expressed in *Arabidopsis* the mitochondrial form is higher expressed in roots than in leaves and the plastidic is expressed in an opposit manner. In contrast, the *Arabidopsis* chloroplastic isoform isolated by Hell et al. (1994) is expressed in similar amounts in leaves and roots and might indicate a functional difference between both chloroplastic isoforms. An increased expression was observed for cysteine synthase isoforms upon sulfur limitation as described for the cytosolic and chloroplastic isoforms from *Arabidopsis* (Hell et al., 1994) and spinach (Takahashi and Saito, 1996). Accordingly sulfur depletion resulted in consequence in a two-fold increase in cysteine synthase activity as described by Schmidt and Jäger (1992). This indicates that cysteine synthase activity is regulated on transcription level. Furthermore, we could show that *AtCS-B* expression occurs in a light dependent manner as it is described for nuclear encoded plastid localized enzymes (Gallagher et al., 1985).

Our analyses of *AtCS-C* and *-B* demonstrate that these genes encode mitochondrial and plastidic forms of cysteine synthase. This conclusion is based on the ability of *in vitro* translation products of *AtCS-C* and *AtCS-B* to enter isolated intact mitochondria and chloroplasts (data not shown), respectively, *in vitro* (Fig. 4) and is supported by analyses of transgenic tobacco plants expressing the mitochondrial cysteine synthase fused to a reporter c-myc epitope. Western blot analyses of transgenic plants expressing AtCS-C/c-myc I revealed translocation into mitochondria while only a minor amount was detected in plastidic fractions, probably due to contamination, supporting data based on *in vitro* import studies of *in vitro* translation products into isolated mitochondria. In contrast, transgenic plants expressing a truncated cysteine synthase cDNA/c-myc fusion did not accumulate the protein in mitochondria but rather in chloroplasts (Fig. 5c). FBPase and ANT antibodies were used to evaluate the purity of the subcellular fractions showing enrichment of the fractions for chloroplasts and mitochondria, respectively.

Current understanding of organellar targeting suggests that when multiple molecular forms of an enzyme are located in different subcellular compartments, each isoform is encoded by a separate nuclear gene. Examples of this include, e.g., the cysteine synthase (Saito et al., 1993, 1994, 1995), malate dehydrogenase (Gietl, 1992), fructose 1,6-bisphosphatase (Raines et al., 1988; Hur et al., 1992) and glyceraldehyde-3-phosphate dehydrogenase (Brinkmann et al., 1989). The possibility of a cotargeting to both mitochondria and chloroplasts mediated by one transit peptide has only been described by Creissen et al. (1995), reporting the simultaneous targeting of pea glutathione reductase to chloroplasts and mitochondria. In the case of glutathione reductase the enzyme is encoded by a single nuclear gene and yet

the enzyme is distributed between chloroplasts, mitochondria and the cytosol. Cysteine synthase in contrast is encoded by three different genes (Hell et al., 1994; Hesse and Altmann, 1995). The high degree of homology found between the presequences of the mitochondrial and chloroplastic isoform allows speculations as to whether or not the mitochondrial form has the ability to be imported by chloroplasts *in vivo*, too. It is not clear whether these homologies have a biological importance *in vivo*. Despite the functional destinction of the mature proteins of both isoforms they are phylogenetically highly related, and thus, grouped into the family of the plastidic CSase isoforms (CS-B). Based on the chimaeric N-terminal structure of the mitochondrial targeting sequence of AtCS-C we speculate that this contradicting feature has been acquired recently in evolution via a duplication of a nuclear localized plastidic gene of *AtCS* due to a fusion to a mitochondrial targeting sequence of *Arabidopsis thaliana*. It has to shown whether AtCS-C represents a single class of mitochondrial isoforms probably complementing the loss of the original mitochondrial isoform or the presence of an additional mitochondrial isoform in Arabidopsis. It remains also to be seen whether CSase C can be imported by chloroplasts under specific conditions, such as those occuring under, e.g. methionine starvation.

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