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An *in vivo* internal deletion in the N-terminus region of Arabidopsis cystathionine γ-synthase results in CGS expression that is insensitive to methionine

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Summary

Cystathionine γ-synthase (CGS), the first enzyme of methionine biosynthesis in higher plants, plays an important role in the biosynthesis pathway and in regulating methionine metabolism in plant cells. In response to methionine, the expression of this enzyme is regulated via amino acid sequences located in its N-terminal. Here, using reverse transcription PCR and ribonuclease protection analysis, we demonstrate that, in addition to the full-length CGS transcript, a deleted form exists in Arabidopsis. The deleted transcript of CGS that lacks 90 or 87 nt located internally in the regulatory N-terminal region of CGS maintains the reading frame of the protein. Its association with polyribosomes indicates that this deleted form of CGS is translated. In order to study the function of this deleted form of CGS, we overexpressed it in transgenic tobacco plants. We found that the transgenic plants engineered to express only the deleted form of CGS accumulated methionine to a much higher level than those that expressed the full-length CGS. Furthermore, *in vitro* feeding experiments revealed that the deleted form of CGS is not subject to feedback regulation by methionine, as reported for the full-length transcript. Therefore, although most likely produced from the full-length CGS, the transcript of the deleted form is insensitive to methionine application and its expression may be important for maintaining methionine metabolism even in the presence of a high level of methionine.

Keywords: methionine synthesis, cystathionine γ-synthase, mRNA stability, methionine metabolism, transgenic plants, sulfur amino acids.

Introduction

The sulfur-containing amino acid methionine is an essential amino acid in animal nutrition. Apart from its role as a protein constituent and its central function in initiating mRNA translation, methionine indirectly regulates a variety of cellular processes as the precursor of *S*-adenosyl methionine (SAM), the primary biological methyl group donor. SAM is also the precursor of plant metabolites such as ethylene, polyamines, biotin and the Fe-chelator mugineic acid (Droux *et al*., 2000; Ma *et al*., 1995; Sun, 1998). Methionine also serves as a donor for secondary metabolites through *S*-methyl methionine (SMM; Mudd and Datko, 1990). Because methionine biosynthesis is so central to cell physiology, it is subject to complex regulatory control. Elucidation of the mechanisms underlying this regulation is currently an important challenge.

The amount of methionine in a plant cell is regulated by the level of its catabolism, by the level of the last enzyme of the threonine biosynthesis pathway, threonine synthase, but mainly by the level of the first enzyme of the methionine biosynthesis pathway, cystathionine γ-synthase (CGS; Amir *et al*., 2002; Avraham and Amir, 2005; Bartlem *et al*., 2000; Hesse and Hoefgen, 2003; Kim *et al*., 2002; Lee *et al*., 2005; Onouchi *et al*., 2004; Ravanel *et al*., 1998a,b; Zeh *et al*., 2001). Studies conducted in Arabidopsis revealed that the activity of CGS, which is located in the chloroplast, is not regulated by classical feedback inhibition (Ravanel *et al*.,

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1998a,b). Instead, both the transcript and protein levels of CGS are regulated indirectly by methionine via the metabolite of methionine, SAM (Chiba *et al.*, 1999, 2003; Onouchi *et al.*, 2005). Unlike bacterial CGS enzymes, the mature plant CGS (without its plastid transit peptides) contains an additional region of approximately 100 amino acids in the N-terminus that is not essential for the catalytic activity of this enzyme (Hacham *et al.*, 2002). A subdomain of this region (termed MTO1 for methionine over-accumulation), which is conserved in the CGS proteins of all plant species, is apparently active in downregulation of its own mRNA and mediates the ability of SAM to control CGS levels (Chiba *et al.*, 1999, 2003; Onouchi *et al.*, 2005). A model was proposed in which the regulation occurs during CGS mRNA translation when the nascent polypeptide of CGS and its mRNA are in close proximity (Chiba *et al.*, 1999, 2003; Lambein *et al.*, 2003; Onouchi *et al.*, 2004). Consistent with this model, it was demonstrated recently that SAM induces temporal translation elongation arrest which precedes the formation of a degradation intermediate of CGS mRNA, whose 5′ end is next to the 5′ edge of the stalled ribosome (Onouchi *et al.*, 2005). Thus, a crucial control point for methionine synthesis in the Arabidopsis plant cell is the amount of CGS transcript.

Whether or not this autoregulation mechanism for post-transcriptional regulation of CGS expression exists in other plant species is still an open question. In potatoes (*Solanum tuberosum*), for example, the transcript level of CGS is not modulated by methionine despite the fact that the MTO1 region in potato CGS is highly conserved compared with that of Arabidopsis (Hesse and Hoefgen, 2003; Kreft *et al.*, 2003; Zeh *et al.*, 2001). This observation suggests that potato is missing some elements required for the post-transcriptional CGS regulation to occur and that the MTO1 region is not sufficient. Therefore, it appears that either other sequences outside the MTO1 region or additional *trans*-acting elements are involved in this regulation of CGS transcript level.

In this work, we report the existence of another control element besides the MTO1 sequence that affects the transcript level of Arabidopsis CGS. While cloning the CGS transcript either via cDNA libraries or by reverse-transcription PCR, we found that in addition to the characterized transcript there is another CGS product. This second transcript contains an internal 90-nt deletion at the N-terminal end. Ribonuclease protection analysis confirmed the presence of this transcript in different organs of the Arabidopsis plant. Its association with polyribosomes suggests that this form of CGS is translated. We found that transgenic plants overexpressing the 90-nt deleted form of CGS display a significantly higher level of methionine than plants overexpressing full-length CGS. When methionine was applied to these transgenic plants, those harboring the deleted transcript failed to respond to methionine exposure. Taken together, these results suggest that an additional transcript of CGS exists in Arabidopsis that is less responsive to methionine levels.

Results

Identification of Arabidopsis CGS transcript containing an internal deletion

When the cDNA of Arabidopsis CGS (AtCGS) was amplified using RNA extracted from different organs and from a flower library, two DNA bands were obtained. The higher band was the expected size for the full-length cDNA encoding the mature CGS enzyme, while the second band was about 0.1 kb shorter (Figure 1a). The sequence of the longer DNA was identical to full-length CGS (Genbank accession number U43709). The sequence of the shorter band proved also to be identical to CGS except for a deletion of 90 nt, between nucleotides 296 and 386, downstream of the ATG translation initiator codon (Figure 1b; Genbank accession number AY545074). Screening of the gene data bank revealed the presence of two expressed sequence tags (ESTs) (accession numbers CF651872 and T22044) that have the same sequence as the shorter band. A sequencing analysis of 24 shorter PCR products revealed that the deletion is either 90 nt (14 products) or 87 nt (10 products; Figure 1b). The two deleted forms do not disrupt the open reading frame of the protein and are predicted to encode AtCGS proteins that lack 30 or 29 amino acids. We next asked whether or not these deleted forms of the AtCGS protein produce active enzymes. We made use of the *Escherichia coli* strain *metB* which is deficient in CGS activity and thus is unable to grow on methionine-deficient medium. We found that, when either AtCGS deleted form was introduced into this strain, growth on methionine-deficient medium was restored (Figure 1c). In summary, these experiments reveal the existence of AtCGS transcripts that contain deletions of either 87 or 90 nt and show that their putative encoded proteins are catalytically active. Next we characterized in more detail the deleted transcripts.

The deleted region is located at the N-terminal region of AtCGS (Figure 1d), which shares no homology with the bacterial enzymes and is not essential for the catalytic activity of AtCGS (Hacham *et al.*, 2002). However, the N-terminal region is important for regulating methionine metabolism and harbors the MTO1 region which regulates the AtCGS transcript level (Chiba *et al.*, 1999; Hacham *et al.*, 2002). A single functional *CGS* gene, located on chromosome 3, exists in the Arabidopsis genome. In addition, a putative pseudogene is located on chromosome 1. However, even if the gene located on chromosome 1 is transcribed, it could not be the template of the deleted CGS transcripts, because it lacks the entire N-terminal region. This implies that the deleted CGS transcripts must be produced by post-

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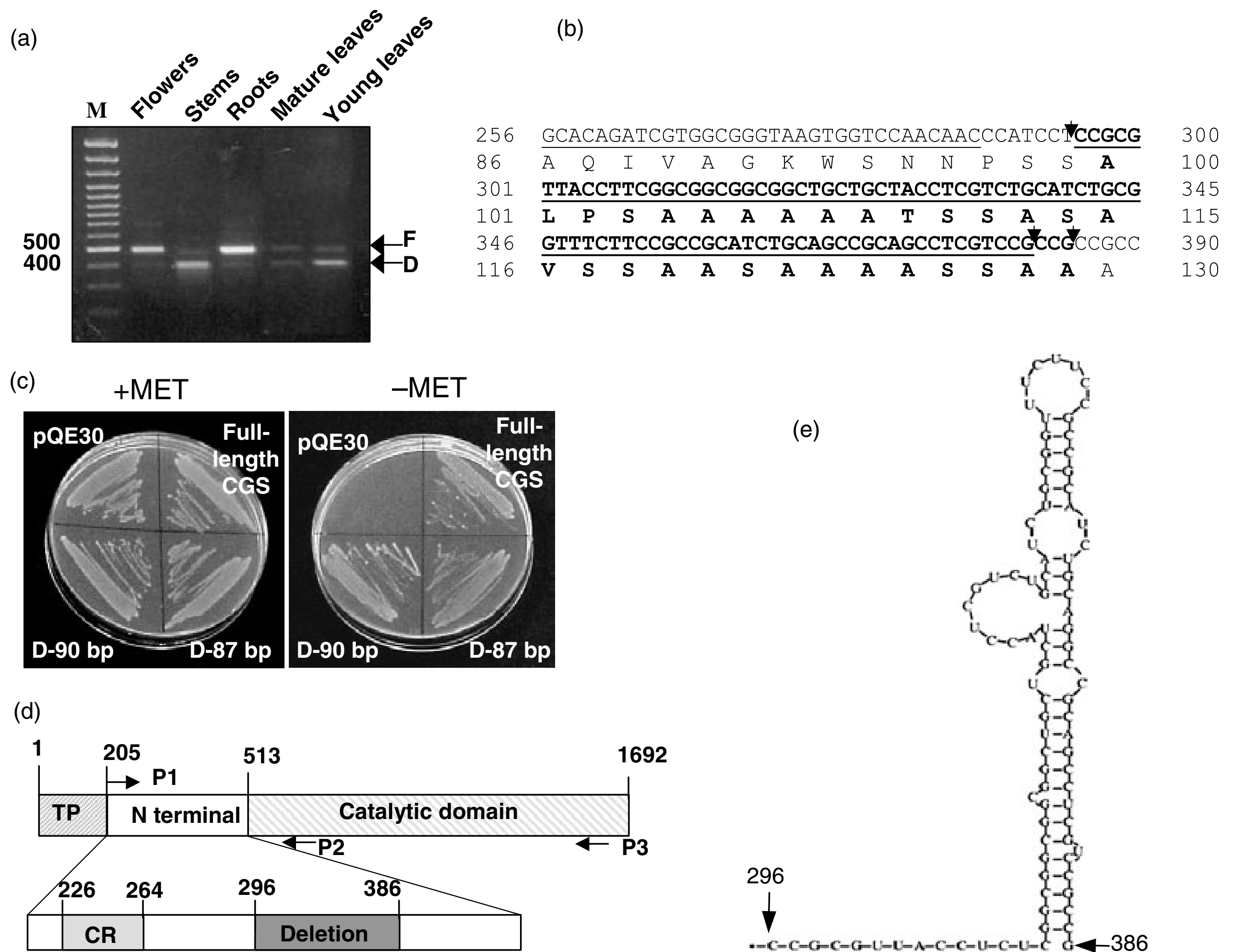

Figure 1. Two forms of CGS mRNAs are present in Arabidopsis.

(a) Reverse transcription PCR using primers P1 and P2 (shown in d) was performed on Arabidopsis RNA samples isolated from different organs. The amplification product of 483 nt derived from the full-length CGS (F) and the product with the deletion of 90 or 87 nt (D) are indicated. M, the 100-nt ladder marker.

(b) The nucleotide and amino acid sequence of the deleted region. The nucleotides and amino acids are numbered from the first ATG and methionine, respectively. Arrowheads indicate the positions of the 5′ start and the 3′ end-points of the deletion. The 87-nt deletion is indicated by bold, underlined letters, while the 90-nt deletion is indicated by bold letters. The part of the sequence that is the conserved region, containing MTO1, is indicated by underlined letters.

(c) Functional complementation of CGS-deficient *Escherichia coli* mutant LE392 with Arabidopsis full-length CGS and the two deleted forms of 90 nt (D-90) and 87 nt (D-87) cDNAs. The plasmid pQE30 (as control) and the same plasmid containing each of the three forms of CGS were transformed into the *E. coli* mutant. The transformed bacteria were grown at 37°C on M9 minimal medium supplemented with 40 μg ml^{-1} methionine (+Met) or without methionine (–Met)

(d) The Arabidopsis mRNA of CGS is shown schematically. TP, the chloroplast-targeting transit peptide; CR, the conserved region that includes the MTO1 domain; deletion, the deleted region described in this work; catalytic domain, the catalytic part of CGS that is highly homologous to bacteria CGSs. The locations of PCR primers P1 and P2 used for the amplification shown in (a) are presented. Nucleotides are numbered from the ATG initiator codon.

(e) Prediction of secondary RNA structure for the 90-nt deleted region. The predicted calculated ΔG of this stem-loop structure is –43 Kcal mol^{-1}.

transcriptional modification of the full-length CGS transcript. The deleted region is located within the first exon of the CGS transcript and is not flanked by any known consensus intron/exon boundary sequences, suggesting that the shorter transcript is not a result of consensus alternative splicing. The deleted region is characterized by a high content of the amino acids alanine and serine (26 out of 30 amino acids; Figure 1b). At the transcript level, it is highly enriched for G-C content (over 70%), suggesting a possible secondary structure. Indeed, two computer programs predicted the formation of a relatively long and stable stem-loop structure, with a ΔG^0 value of –43 Kcal mol^{-1} (Figure 1e). Other 90-nt-long segments taken from random parts of the CGS transcript were predicted to form less stable secondary structures with ΔG^0 values ranging between –15 and –25 Kcal mol^{-1}.

As the reading frame of the CGS protein is not disturbed by the deletion, and the deleted region is located in the N-terminal region which is important for controlling expression of the gene, we hypothesized that a deleted form of CGS

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could be involved in the control of CGS activity and/or its expression and therefore could affect methionine metabolism. To investigate this hypothesis further, we decided to express the deleted and full-length forms of Arabidopsis CGS in transgenic tobacco plants (*Nicotania tabacum*) and analyze the effect of expressing the corresponding proteins on methionine metabolism.

Producing transgenic tobacco plants expressing full-length and deleted AtCGS

To examine whether the deleted AtCGS affects methionine metabolism, we transformed Arabidopsis and tobacco plants with constructs containing the full-length or 90-nt deleted Arabidopsis CGS cDNAs. A DNA-encoded plastid transit peptide of the pea (*Pisum sativum*) rbcS-3A was fused in-frame in order to direct the proteins to the chloroplast (Figure 2a). Additionally, three copies of the hemaglutinin (HA) epitope tag were fused to the 3′ region of the CGS open reading frames (Figure 2a) to enable detection of the proteins in the transgenic plants by immunoblotting. Thirty independent T_0 transgenic Arabidopsis and tobacco lines expressing each of the CGS constructs were selected and transferred to the greenhouse for further growth. The transgenic Arabidopsis plants showed very low levels of wild-type and transgenic CGS gene expression, possibly as a result of co-suppression (data not shown). Therefore, we studied further the heterologous system of the transgenic tobacco plants in which both constructs were well expressed (see below). AtCGS expression in vegetative tissues of the T_0 plants was assayed by Western blot analysis using anti-HA monoclonal and anti-CGS antibodies. Fifteen lines displaying the highest expression levels for either full-length or deleted AtCGS were selected. Following selfing of the selected T_0 tobacco lines, the T_1 progeny obtained were seeded on kanamycin-containing medium in order to analyze transgene segregation. T_1 progeny sprouted at a 3:1 ratio, indicating that the transgene behaves as a single dominant locus.

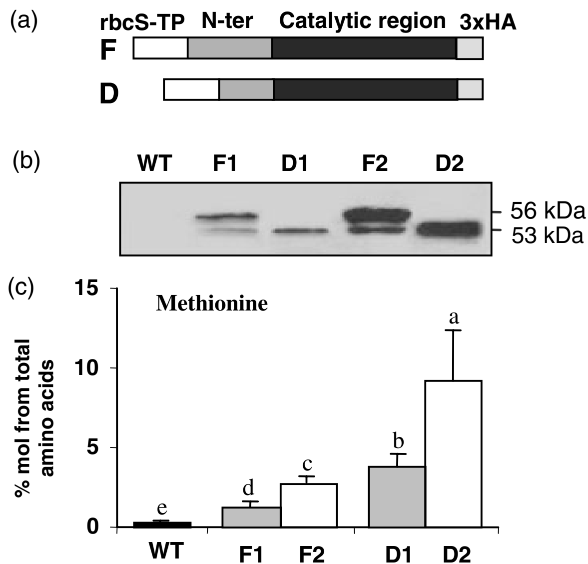

Figure 2. Transgenic tobacco plants expressing the deleted Arabidopsis CGS produce elevated amounts of methionine.
(a) The constructs used to express the full-length or deleted Arabidopsis CGS in tobacco plants. RbcS-TP, the transit peptide of the Rubisco small subunit 3A of pea (*Pisum sativum*), was used instead of the endogenous peptide; N-ter, the N-terminal region of CGS where the deletion is found; 3 × HA, the sequence encoding three hemaglutinins, was added in-frame at the C-terminus of the protein.
(b) Immunoblot analysis of proteins extracted from non-transformed tobacco (WT), two transgenic plants expressing full-length CGS (F1 and F2) and two transgenic plants expressing the deleted form of CGS (D1 and D2). Proteins (10 μg) extracted from 8-week-old plants were fractionated by SDS-PAGE and subjected to immunoblot analysis using antiserum against the HA epitope tag.
(c) Soluble methionine levels in 8-week-old wild-type plants (WT) and transgenic tobacco plants. The methionine level, as detected by HPLC, is presented as the mol. % of total free amino acids. The total amino acid content did not differ significantly between wild-type and transgenic lines. The data are presented as the mean ± standard deviation of five plants per line.

While the phenotype of the T_0 transgenic plants expressing the deleted and full-length AtCGS versions was normal, the T_1 plants expressing high levels of either full-length or deleted AtCGS displayed an abnormal phenotype that could be recognized easily after four weeks of growth. The abnormal phenotype included stunted growth, slow development rate, narrow and curly leaves, and a high emission rate of dimethylsulfide. It resembled the phenotype of tobacco plants expressing the Arabidopsis CGS lacking the whole N-terminal region (Hacham *et al.*, 2002). The phenotype observed in the T_1 plants raised the possibility that the transgenes are causing changes in the regulation of methionine metabolism. Therefore, we analyzed methionine levels as well as the levels of other amino acids in the transgenic plants.

In order to compare in a controlled manner the methionine contents of plants expressing full-length versus deleted AtCGS, lines were paired so that transgenic plants expressing similar amounts of the full-length and deleted forms of AtCGS were evaluated side by side. Figure 2(b) illustrates an example of two such pairs. As expected, no anti-HA immunoreactive protein band was detected in untransformed plants (Figure 2b, lane 1). However, transgenic plants expressing the full-length and deleted forms of AtCGS displayed 56- and 53-kDa bands, respectively (the HA tag contributes 3 kDa; Figure 2b). In addition, and as previously shown (Hacham *et al.*, 2002), plants expressing full-length AtCGS also exhibited a second band migrating more rapidly, having an estimated size of 53 kDa (Figure 2b). The lower band co-migrated with the band found in the transgenic plants expressing the deleted form, suggesting that the deleted form could be produced from the full-length cDNA of Arabidopsis CGS. The intensity of the lower band varied significantly depending on the

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Table 1 Contents of aspartate-related amino acids in wild-type and transgenic plants expressing the full-length and deleted forms of Arabidopsis CGS

Amino acid (% mol.)	Wild-type	Full-length CGS	Deleted CGS
Aspartate	7.2 ± 3.9	5.3 ± 3.4	9.3 ± 1.9
Threonine	3.2 ± 0.8	4.0 ± 0.9	3.7 ± 0.3
Lysine	0.9 ± 0.4	1.0 ± 0.4	0.8 ± 0.1
Isoleucine	0.8 ± 0.48	1.0 ± 0.7	1.2 ± 0.2

The amounts of amino acids were calculated from total amino acids as detected by HPLC and are given in mol. %. The total amino acid content did not differ significantly between wild-type and transgenic lines. Eight plants of each type were analyzed and the data are presented as the mean ± standard deviation.

age of the plant or tissue type from which the extract was derived.

Transgenic plants expressing the deleted form of CGS had higher methionine levels

When the T_1 plants were analyzed for the quantity of free methionine, significantly higher levels of free methionine were found in plants expressing the deleted form of CGS than in plants expressing the full-length form [shown for two pairs in Figure 2(c)]. In general, the amount of methionine was proportional to the transgenic protein level.

Methionine belongs to the aspartate family of amino acids. Therefore, we studied whether the dysfunctional methionine regulation in these transgenic plants was associated with abnormal levels of other amino acids belonging to this family. No significant difference in the levels of amino acids belonging to the aspartate family was observed between the wild type and two lines of transgenic plants (Table 1). Similar results were obtained with various other amino acids, with the exception of glycine, in which a decrease from 13 to 5 mol. % was detected.

Taken together, our results suggest that the natural deletion of 30 amino acids in the N-terminal region of AtCGS leads to altered methionine levels. Therefore we propose that this 30 amino acid region is a regulatory element controlling CGS expression. In order to investigate this hypothesis, and because no conservative splicing elements are apparent in this region, we set out to confirm whether or not the deleted CGS transcript is indeed produced in Arabidopsis and in transgenic tobacco plants expressing the full-length AtCGS.

The deleted form of CGS is present in vivo *in Arabidopsis and in transgenic tobacco plants*

As described above, two PCR products were found using RT-PCR (Figure 1). However, it is well-known that this method can create artifacts, especially when a secondary structure such as a stable stem-loop structure is present. Thus, to confirm that the deleted form of CGS is indeed present *in vivo* in the Arabiodosis plant, we assayed for this transcript using the ribonuclease protection assay. Two [^{32}P]-labeled antisense RNA probes corresponding to the 5′ region of the CGS transcript were produced (Figure 3). Each probe was hybridized with Arabidopsis RNA or with RNA isolated from transgenic tobacco plants. Following digestion with RNase T1, the protected products were separated on denaturing gels and analyzed along with RNA of known lengths as markers. The first probe was designed to hybridize to the sequence between the first ATG of CGS and the center of the deleted region (Figure 3a, bottom). Using this probe with total RNA derived from Arabidopsis we observed, as expected, two protected fragments of 353 nt (representing the full-length CGS) and 296 nt (representing the deleted transcript; Figure 3a). Analysis of RNA isolated from transgenic tobacco plants expressing the full-length CGS revealed two protected transcripts of 148 and 91 nt. These two transcripts correspond to the full-length and deleted transcripts, respectively, each lacking the first 205 nt encoding the chloroplast transit peptide that had been replaced by the pea rbs 3A transit peptide in the transgenic plants. As expected, when we analyzed RNA purified from transgenic plants expressing the deleted form of CGS, we detected only the protected band of 91 nt (Figure 3a). The endogenous tobacco CGS transcript was not detected by the probe directed at Arabidopsis CGS. The lack of any protected bands when a control ribonuclease protection experiment was performed using yeast tRNA shows the specificity and accuracy of this analysis. Together, these results indicate that the deleted form of the CGS transcript is indeed produced in Arabidopsis and in transgenic tobacco expressing full-length AtCGS. However, it remains possible that cleavage of the AtCGS transcript at nucleotide 296 by an endoribonuclease has produced two cleavage products, whereby the proximal one is detected by probe 421 (Figure 3a). Therefore, in order to exclude this possibility, a second probe was designed and used in the RNase protection assay.

The second probe, probe 504, illustrated in Figure 3(b), was derived from the deleted cDNA and spanned the first 526 nt starting from the first ATG. Protected bands of 436, 296 and 140 nt were observed in RNA extracted from Arabidopsis (Figure 3b). The 140- and 296-nt bands correspond to two protected fragments expected from the full-length transcript. The 436-nt protected fragment could only be obtained by protecting a deleted transcript, proving that such a transcript is indeed present in Arabidopsis. These three bands were also obtained using RNA isolated from transgenic plants expressing full-length AtCGS. As the CGS in these plants lacks the Arabidopsis transit peptide, the protected fragments representing the deleted form of CGS and the 5′ fragment of the full-length

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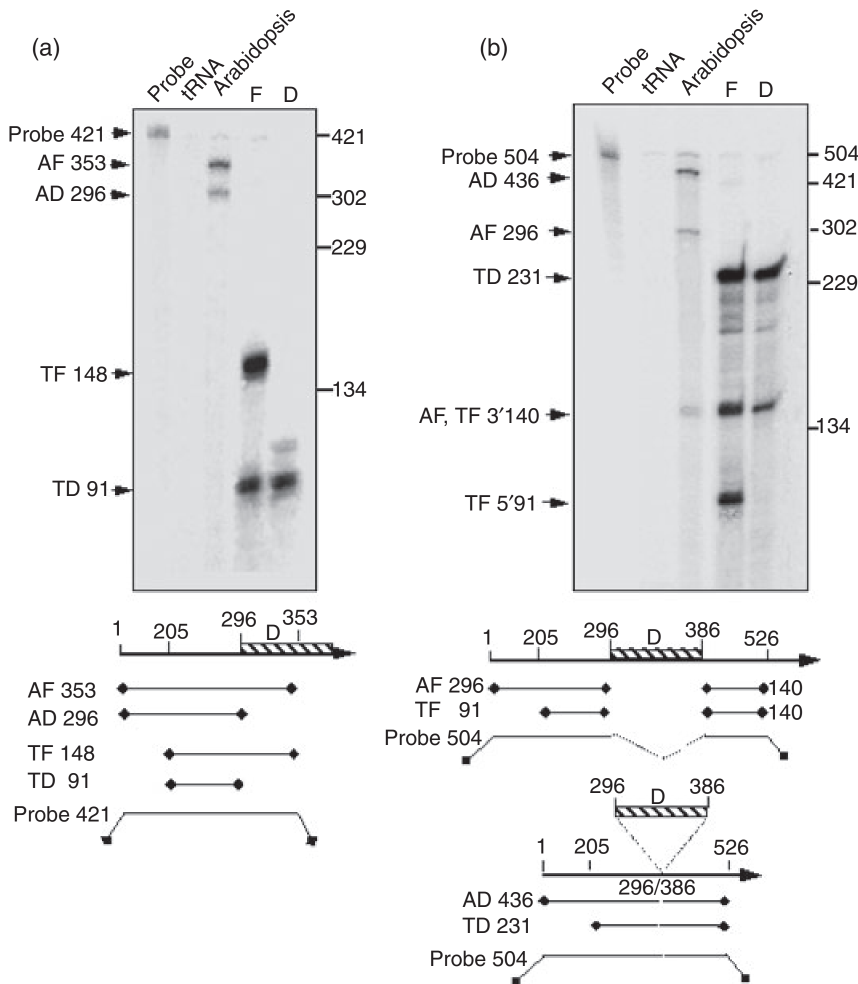

Figure 3. Detection of the deleted CGS mRNA in Arabidopsis and in transgenic tobacco plants.
RNA (20 μg) was isolated from Arabidopsis and transgenic tobacco plants expressing the full-length Arabidopsis CGS (F) or the deleted form (D). The RNA was annealed to uniformly [^{32}P]-labeled probes spanning regions of the CGS, as shown schematically at the bottom of the figure: (a) Using probe 421, which includes the MTO1 domain and part of the deleted region; (b) using probe 504, which includes the N-terminal region but lacks the deletion region. The probes included several nucleotides not related to the CGS sequence on each side in order to separate the full-length probe from the protected RNA fragment. A schematic representation of the CGS mRNA and sizes of the probe and protected fragments are indicated in the schemes below. The RNA was digested with RNase T1 and then analyzed by gel fractionation and autoradiography. A parallel control reaction contained no plant RNA but an equivalent amount of yeast tRNA. The positions of size markers of [^{32}P]-labeled RNAs of known length are indicated on the right. The protected probe fragments corresponding to Arabidopsis full-length CGS (AF), the deleted form of CGS (AD), the full-length *Arabidopsis* CGS expressed in tobacco (TF) and the deleted form of CGS expressed in tobacco (TD) are indicated by arrows on the left. The 5′ end of the Arabidopsis CGS expressed in tobacco is at nucleotide 205.

CGS are shorter (231 and 91 nt, respectively; Figure 3b). When the RNase protection assay was performed on RNA extracted from tobacco plants expressing the deleted AtCGS, two bands were observed, one with the expected size of 231 nt, representing the deleted form, and another of 140 nt. The presence of the latter fragment could not be explained by the protection of fragments expected from the full-length or deleted transcripts. The appearance of this band suggested that in these transgenic plants, in addition to the production of the deleted form of the CGS transcript, another truncated transcript is formed, likely by an endonucleolytic cleavage at position 386 (Figure 3b, bottom). We assume that the proximal cleavage product is degraded rapidly and thus only the distal cleavage product is accumulated, resulting in an extra 140-nt protected fragment.

The cDNA analysis together with the ribonuclease protection assays clearly demonstrated that a deleted version of CGS mRNA is produced in Arabidopsis and in the tobacco transgenic plants expressing the full-length Arabidopsis CGS transcript. In addition, it appears that a cleavage occurs at nucleotide 386, resulting in a third transcript (starting at nucleotide 386 to the end of the CGS).

The deleted form of the CGS transcript is associated with polysomes

Following the observation that the deleted transcript form of CGS is indeed present in Arabidopsis, we asked whether or not this form is translated by examining its association with polysomes. To this end, polysomal fractions derived from total cell lysates produced in non-ionic detergents and cyclohexamide (to prevent runoff translation) were sedimented in analytical sucrose gradients. Extracts of leaves of transgenic tobacco expressing the full-length and deleted AtCGS transcripts and Arabidopsis leaves were fractionated, and the RNA purified from the different fractions was analyzed by ribonuclease protection assay for the presence of the full-length and the deleted forms of CGS. Figure 4(a) displays the distribution of the ribosomal RNA between the different fractions as

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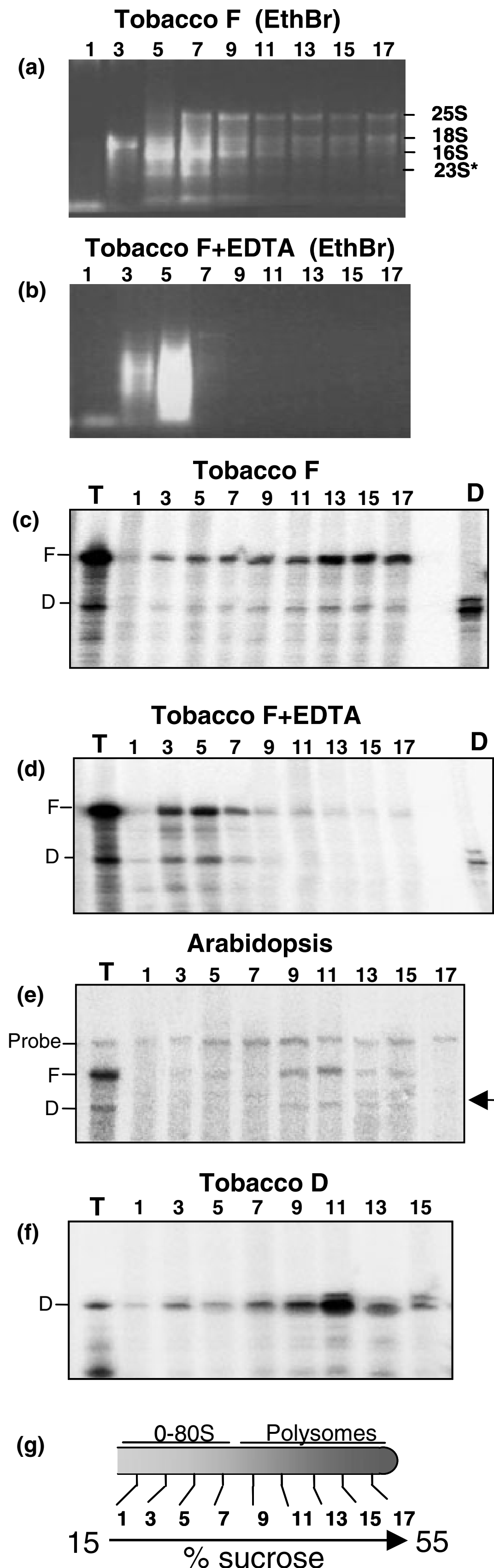

revealed by ethidium bromide staining. Fractions 1–7 harbor the RNA that is not associated with polysomes as well as the RNA associated with monosomes, while fractions 8–17 contain the polysomes (Figure 4a,g). To verify that the rRNAs observed in fractions 8–17 are derived from polysomes, a control fractionation was carried out whereby cell lysates were treated with EDTA and fractionated through EDTA-containing gradients. EDTA causes dissociation of polysomes into monosomes (Rott *et al.*, 1999). Indeed, EDTA treatment resulted in all the rRNAs being observed in fractions 1–7 and disappearing from fractions 8 to 17 (Figure 4b). Having established the capability of our method to discern polysomal association, we analyzed the distribution of the full-length and the deleted CGS transcripts between the monosomes (fractions 1–7) and polysomes (fractions 8–17). Most of the two transcripts were found in fractions 9–17 and are therefore polysome-associated (Figure 4c,e,f). This was true for the transgenic tobacco expressing the full-length or the deleted form of AtCGS, as well as for the endogenous CGS transcripts in Arabidopsis. Because of the low abundance of the deleted form in Arabidopsis, it was difficult to detect its signal using this analysis. Nevertheless, as shown in Figure 4e, it is clear that this transcript is associated with polysomes. As with the ribosomal RNA, EDTA treatment resulted in the relocation of the CGS transcripts to the non-polysomal fractions [shown in Figure 4(d) for the transgenic tobacco expressing the full-length CGS].

Taking our data as a whole, we propose that the deleted form of the CGS transcript is translated and that

Figure 4. Association of the transcripts of the full-length and the deleted forms of CGS with polysomes.
Polysomal fractions derived from total cell lysates prepared from leaves of transgenic tobacco (10 weeks old), and Arabidopsis (30 days old) plants were sedimented through analytical 15–55% sucrose gradients. Seventeen fractions were collected. RNA purified from the lysate of total leaf (T) as well as from the various fractions were assayed from tobacco plants expressing the full-length (F) and the deleted (D) forms of CGS, using probe 421 (described in Figure 3a) for the ribonuclease protection assay.
(a) Ethidium bromide (EthBr) staining of RNA purified from the fractions and fractionated on a denaturing agarose gel to reveal the distribution of the rRNA. The pattern for tobacco plant F is presented (similar gels were obtained for the other plants but are not shown).
(b) As for (a), but the fractions were treated with EDTA as described in the Experimental procedures in order to disrupt the polysomes.
(c) Ribonuclease protection analysis of the transgenic tobacco plant expressing the Arabidopsis full-length CGS (F). In the lane labeled D, analysis of plants expressing the deleted form of CGS was performed as a marker for the deleted (D) CGS transcript.
(d) As for (c), but the extract was treated with EDTA before the sucrose gradient step.
(e) Ribonuclease protection analysis of extract obtained from Arabidopsis plants. An arrow indicates the D transcript.
(f) Ribonuclease analysis of transgenic tobacco plants expressing the deleted form of Arabidopsis CGS.
(g) A schematic representation of the sucrose gradient fractionation displaying the location of the fractions and the distribution of the polysomes.

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the corresponding protein is produced in transgenic tobacco plants expressing the full-length and the deleted AtCGS, and is also produced endogenously in Arabidopsis plants.

The deleted form of the CGS transcript is found in different organs of the Arabidopsis plant

Methionine content varies in different organs of Arabidopsis (Bartlem *et al.*, 2000; Kim *et al.*, 2002). Therefore, we wished to examine whether the level of the deleted form of CGS varies in different organs. To this end, the RNase protection assay was performed on RNA extracted from different Arabidopsis organs using probe 421 (Figure 3a). This experiment revealed, as indicated by the appearance of the protected fragment of 296 nt, the presence of the deleted form of CGS in all organs examined, including flowers, stems, mature and young leaves, and roots (Figure 5a). The proportion of the deleted form of CGS mRNA to the full-length form was similar in all of the tested organs, indicating limited, if any, control at the formation of the deleted CGS transcript (Figure 5a).

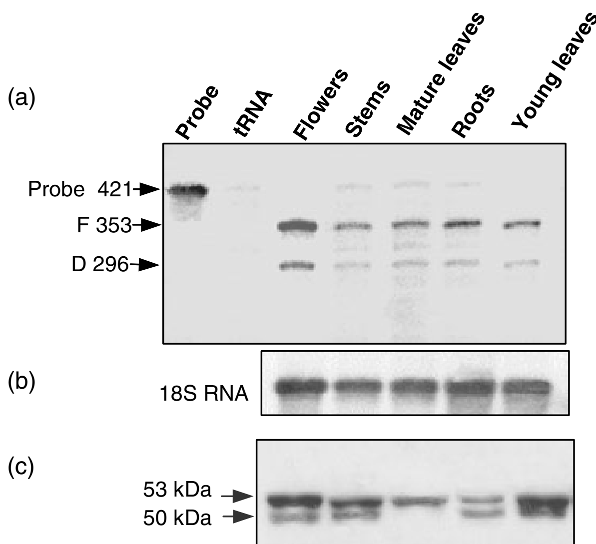

Figure 5. The deleted form of CGS is expressed differentially in Arabidopsis organs.
(a) RNA isolated from Arabidopsis organs as shown at the top of the figure and subjected to ribonuclease protection assay using the uniformly labeled probe 421 described in Figure 3a. A parallel control reaction contained no plant RNA but an equivalent amount of yeast tRNA. The protected fragments corresponding to the full-length CGS (F) and the deleted CGS (D) are indicated.
(b) RNA blots were hybridized with 18S rRNA probe as a control for equal loading.
(c) Western blot analysis of proteins extracted from the same organs. Protein extracts (20 μg) were analyzed by immunoblotting using antiserum against the Arabidopsis CGS. This antiserum does not react with the tobacco CGS.

Next, the relative amount of CGS protein was analyzed in these organs by immunoblotting using specific CGS antibodies. As described for the transgenic tobacco plants [this study and Hacham *et al.* (2002)] and for Arabidopsis (Ravanel *et al.*, 1998a), two bands were detected: the upper band migrates with the expected size of the natural mature Arabidopsis CGS (53 kDa; Ravanel *et al.*, 1998a), and the second-band migrates more rapidly, having an estimated size of 50 kDa. The appearance of two immunoreactive bands supports the possibility discussed earlier that the lower band is the translation product of the deleted CGS transcript (Figure 2b). The intensity of the lower band varied between repeat experiments and thus several analyses were performed on each organ (a representative immunoblot is shown in Figure 5c). The relative abundance of the two immunoreactive bands varied for different organs. For example, two bands appeared in young leaves, flowers and stems while only the upper band was present in mature leaves. The two bands were of similar intensity in roots. Taken together, these results indicate that there is poor correlation between RNA and protein levels of the CGS deleted transcript and further suggest that these two forms of CGS are regulated at translational or post-translational levels.

Methionine modulates the transcript and protein levels of full-length CGS but not those of the deleted form

Methionine modulates the transcript and protein levels of full-length CGS in Arabidopsis (Chiba *et al.*, 1999; Onouchi *et al.*, 2004, 2005). Analysis of transgenic tobacco plants expressing the deleted form of CGS revealed elevated amounts of methionine compared with those expressing the full-length form (Figure 2c). In order to determine if the deleted CGS is subject to modulation of RNA and protein levels in a similar manner to the full-length CGS, freshly detached leaves of transgenic tobacco plants overexpressing either the full-length or the deleted CGS form were fed with 5 mM methionine for 4 h. The amounts of CGS transcript and protein were then analyzed. The results confirmed that, in transgenic plants engineered to contain full-length AtCGS, the amount of full-length protein was drastically reduced by methionine treatment, as previously reported for Arabidopsis (Chiba *et al.*, 1999; Onouchi *et al.*, 2004, 2005). However, when transgenic plants engineered to express only the deleted AtCGS were examined, no similar reduction in protein or transcript content was observed (Figure 6a).

To further elucidate the apparent differences between the two forms of CGS in their response to methionine, we analyzed these mRNA forms using an *in vitro* coupled transcription/translation system based on wheat germ extracts. This system, when applied to CGS, gave results that reflected the data obtained in the *in vivo* studies (Chiba *et al.*, 2003; Kreft *et al.*, 2003). The intention was to test

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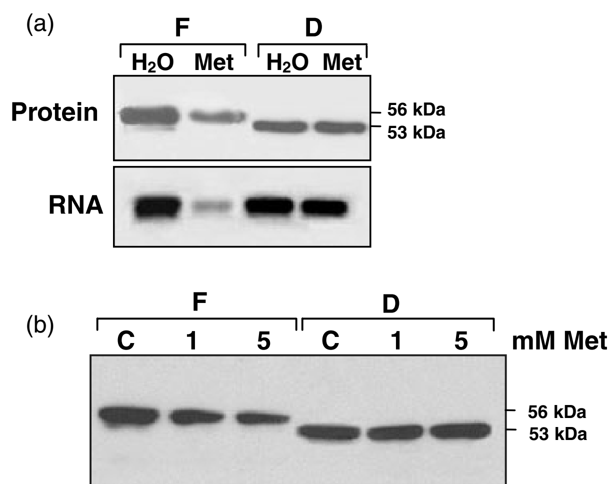

Figure 6. Modulation of expression of the full-length and deleted CGS by methionine.
(a) Effects of exogenously applied methionine content on CGS expression level in detached leaves of transgenic tobacco harboring the Arabidopsis full-length (F) or deleted form of CGS (D). The leaves were freshly cut from 10-week-old greenhouse-grown plants and incubated in water (H_2O), as a control, or with 5 mM methionine (Met) for 4 h. The upper panel shows an immunublot analysis of the amount of transgenic CGS using antiserum against the HA epitope tag that was attached to the Arabidopsis CGS when transformed into tobacco. The lower panel shows analysis of the transcript level of the Arabidopsis CGS by RNA-blot using a gene-specific probe.
(b) Effect of two methionine concentrations on the protein levels of the two forms of CGS. The mRNA levels were produced in a wheat germ *in vitro* coupled transcription/translation system. Samples containing 1 μg of linearized plasmid DNA (pBluescript KS^-) harboring cDNAs coding for the full-length (F) or deleted forms (D) of CGS were incubated in wheat germ extracts containing 0.004 (c, for control), 1 or 5 mM methionine for 90 min at 30°C. The amount of CGS protein was detected by immunoblot assay.

transcript stability that relied on accurately defined methionine concentrations independent of uptake into the plants. cDNAs coding for these two forms of CGS were transcribed/translated in the presence of 1 and 5 mM methionine, and the translated products were subjected to Western blot analysis (Figure 6). As a control, total amino acid containing 4 μM methionine was added to the extract (C in Figure 6b). This low methionine level does not affect the transcript level of the full-length CGS (data not shown) or limit protein synthesis efficiency (Kreft *et al.*, 2003). The protein level of the full-length CGS (F in Figure 6) showed a distinct decrease when methionine was applied, whereas the protein level of the deleted form of CGS (D in Figure 6) was found to be unaffected by methionine within the range of the experimental set-up (Figure 6b). Taken together, these experiments suggest that the deleted form of CGS is not subjected to metabolic control by methionine. In addition, they lead us to propose that the sequence of 90 nt, which is not present in the deleted form of AtCGS, is involved in the modulation of CGS transcript levels by methionine.

Discussion

A new regulatory domain in Arabidopsis CGS

Previous studies have shown that the amount of methionine in the plant cell is controlled mainly by the level of its key biosynthesis enzyme, CGS [reviewed by Amir *et al.* (2002); Hesse and Hoefgen (2003); Hesse *et al.* (2004) and Onouchi *et al.* (2004)]. In Arabidopsis, CGS is regulated at the mRNA stability level via negative feedback mediated by the methionine metabolite, SAM (Chiba *et al.*, 1999, 2003; Onouchi *et al.*, 2005). The amino acid sequence encoded by the MTO1 domain, located within a 5′ conserved region, is responsible for this regulation and acts to destabilize its own mRNA (Chiba *et al.*, 1999; Onouchi *et al.*, 2005). It has been proposed that the regulation occurs during translation when the nascent polypeptide of CGS and its mRNA are in close proximity (Lambein *et al.*, 2003; Onouchi *et al.*, 2005).

In this work, we have shown that, in addition to the MTO1 domain, another regulatory domain is located within the N-terminal regulatory region of the Arabidopsis CGS transcript. This new domain is 90 nt long, located between nucleotides 296 and 386 downstream of the ATG initiator codon, and plays a significant role in modulating the CGS transcript level in response to methionine. The transcript level of the full-length CGS enclosing this region is highly sensitive to methionine application, as previously shown (Chiba *et al.*, 1999, 2003), while the deleted form of the CGS transcript lacking this region was not responsive to this application (Figure 6). This suggests that the 90-nt sequence plays a significant role in regulating the CGS transcript level.

It has been shown previously by Ominato *et al.* (2002) that the conserved region where the MTO1 is located plays an essential role in controlling the downregulation of CGS. By creating deletions within the first exon of CGS and performing transfection experiments, it was found that, when the conserved region was missing, CGS was not downregulated in response to methionine application. In contrast, deletion constructs that retained the conserved region did respond to methionine application (Ominato *et al.*, 2002). These results suggested that the conserved region is necessary and sufficient for regulation. However, the findings described in the present study suggest that the regulation of CGS is more complex as the deleted form of CGS does retain MTO1, and yet is not responsive to methionine application, indicating that these two motifs are necessary for the downregulation of the full-length CGS.

The importance of the deleted 90- or 87-nt sequence in controlling the CGS transcript level was revealed when a form of CGS that lacks this sequence was found in Arabidopsis. RT-PCR and RNase protection assays performed on different Arabidopsis organs demonstrated that this deleted transcript is present *in vivo* in addition to the full-length

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CGS. Feeding experiments using transgenic tobacco leaves overexpressing this deleted form of CGS and *in vitro* studies using the coupled transcription/translation system showed that its transcript level (Figure 6), unlike that of full-length CGS (Figure 6; Chiba *et al.* 1999, 2003), is not responsive to methionine application.

How is the deleted CGS transcript formed?

How the deleted form of the CGS transcript is produced is still an open question. However, on the basis of the results of this study, we suggest that it is generated from the transcript of the full-length CGS. The fact that the sequences of the two forms are identical, except for the deleted 90 or 87 nt, and the finding that only one *CGS* gene that contains the N-terminal region exists in Arabidopsis support this assumption. As no conserved intron/exon-splicing borders are located in this region, formation of the deleted CGS by conventional alternative splicing is not likely.

Two observations lead us to hypothesize that the deletion occurs when the full-length mRNA is translated and associated with ribosomes: (i) the two deleted forms of CGS, lacking either 90 or 87 nt, both maintain the reading frame of full-length CGS; (ii) the transcript level of the deleted form is proportional to that of the full-length form in the different Arabidopsis organs (Figure 5). An alternative mechanism for generating deleted CGS is that it is produced from the pre-mRNA in the nucleus. However, if this were the case, then, in Arabidopsis organs where methionine levels are high, such as stems, mature leaves and flowers (Bartlem *et al.*, 2000; Kim *et al.*, 2002), the deleted CGS transcript level should remain stable while the full-length CGS transcript level should be reduced. However, such differences between the two CGS transcript levels were not observed (Figure 5a), again suggesting that it is unlikely that an alternative splicing mechanism is involved in generating the deleted CGS transcript.

Additional recent evidence supports the assumption that the deleted form can be produced during the translation process. A temporary arrest in the translation process at serine-94 (nucleotide 280 from the first ATG of the mRNA) was demonstrated when the SAM level increased, and a strong correlation between this arrest and mRNA degradation was observed (Onouchi *et al.*, 2005). Using toeprint, the researchers demonstrated two-toeprint signals, the main one located at nucleotide 293 and the second at nucleotide 296 [Figure 6(d) in Onouchi *et al.* (2005)]. The signal at nucleotide 296 is located exactly at the putative cleavage site hypothesized for the formation of the deleted CGS transcript (at the 5′ side) detected in this study [shown by RT-PCR (Figure 1a) and ribonuclease assay; Figure 3a]. The other cleavage site for the formation of the deleted form of CGS is located at nucleotide 386. In our study, we detected a cleavage at this point using the ribonuclease assay in the transgenic plants expressing the deleted form of CGS (Figure 3b). The mobility of this cleavage product band indicated a size of 140 nt, which represents a putative transcript that could be generated by an endonucleolytic cleavage at position 386, which is located at the 3′ side of the deletion region. Following the initial cleavage at nucleotide 386, not all cleaved transcripts are ligated to the 5′ sequence, resulting in the formation of the deleted form of CGS. Thus, the proximal unligated product undergoes rapid degradation while the distal product is accumulated, resulting in the truncated CGS transcript as revealed by the 140-nt protected fragment (Figure 3b). We believe that, during translation arrest and cleavage, at least two cleavage products of the mRNA of CGS are ligated to produce the deleted forms of CGS. We assume that the efficiency of this process (cleavages and ligation) is low, resulting in a low proportion of the deleted form compared to the full-length CGS (Figure 5a). If this is the case, several cleavages must occur within the first exon following the translation arrest, which may lead to the degradation (Onouchi *et al.*, 2005) or formation of the deleted form of CGS (summarized in Figure 7). Further analysis is required to define the nature of each of the CGS truncated forms and to elucidate the role of methionine/SAM in regulating the cleavages that may lead to their formation, as the formation of the deleted form of CGS is apparently not regulated by methionine/SAM levels because it is produced in different organs that harbor various methionine contents in their cells (Figure 5a).

Using ribonuclease protection assays, we have shown that the full-length and deleted transcripts of CGS detected endogenously in Arabidopsis are also produced in transgenic tobacco plants engineered to express full-length Arabidopsis CGS. This indicates that the machinery responsible for creating the deleted transcript is not unique to Arabidopsis and is also active in tobacco.

Translation of the deleted CGS transcript

Based on our observation that both deleted and full-length CGS transcripts are associated with polysomes (Figure 4), we suggest that the deleted form is translated into a protein and, moreover, produces an active enzyme. Several observations support this premise. First, we observe an additional protein band of the expected molecular size for the deleted form (approximately 50 and 53 kDa, respectively) when Western blot analysis is performed on Arabidopsis and transgenic tobacco plants expressing full-length CGS. Secondly, prior to our study, Ravanel *et al.* (1998a) detected two co-purifying bands for Arabidopsis CGS whose estimated sizes were 53 and 50 kDa and confirmed by Edman degradation that both polypeptides possessed the same N-terminal CGS sequence. The authors suggested that the shorter polypeptide resulted from degradation of the full-length protein at its C-terminal end during the purification procedure, but we propose that in

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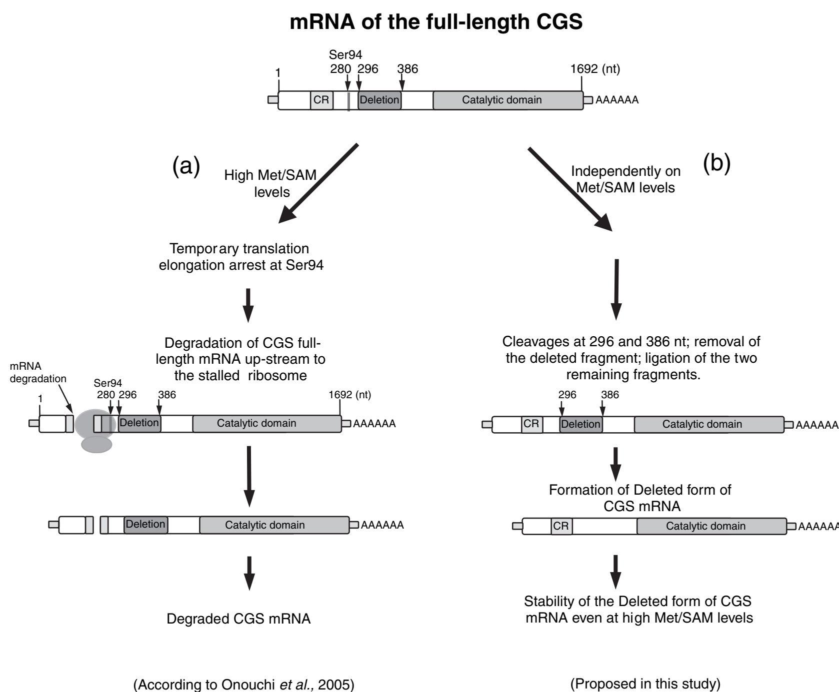

Figure 7. A scheme of the post-transcriptional regulation of the Arabidopsis CGS.
(a) The model proposed by Onouchi *et al.* (2005) for full-length Arabidopsis CGS mRNA, which leads to its degradation when a high level of cellular Met/SAM is present.
(b) The model proposed in the current study for the generation of the deleted CGS form from the full-length CGS independent of Met/SAM levels. The deleted form of the CGS mRNA is possibly produced by putative cleavage at positions 296 and 386 nt, as found by the RNase protection assay (Figure 3) and RT-PCR (Figure 1a,b). One possibility is that the region of the deletion is removed following the cleavages and the remaining two mRNA cleavage products are ligated to create the deleted form of CGS. This form of CGS is not subjected to regulation by a high level of methionine/SAM. CR, the conserved region that includes the MTO1 domain; deletion, the deleted region described in this work; catalytic domain, the catalytic part of CGS that is highly homologous to bacteria CGSs; Ser94, the location where the elongation translation arrest occurs (Onouchi *et al.*, 2005).

fact they were detecting the full-length and the deleted CGS forms (Ravanel *et al.*, 1998a). In the present work, the Arabidopsis CGS clones were fused in-frame to a 3HA epitope tag at their C-terminal region. As two bands were observed when antibodies were used against this tag, we can conclude that this shorter band is not a result of degradation of a C-terminal region. As the data of Ravanel *et al.* (1998a) indicate that generation of their observed shorter CGS form does not involve N-terminus degradation, and as our experiments imply that the C-terminus is intact in the shorter CGS form that we detect, we believe that the 50-kDa band results from the translation of the deleted CGS transcript characterized in this work. Two other observations further support the assumption that the deleted CGS encodes an active enzyme: (i) expression of deleted CGS complemented the methionine metabolism defect of the *E. coli metB* mutant, and (ii) tobacco plants expressing the deleted form of CGS contain significantly higher methionine levels. In summary, all our data, and those of Ravanel *et al.* (1998a), suggest the conclusion that the deleted transcript product is indeed translated to a protein and forms an active enzyme.

What is the function of the deleted CGS form?

We suggest that the functional role of the deleted form of CGS in Arabidopsis is to enable the continued synthesis of methionine irrespective of the methionine/SAM level in cells. This later role for the deleted CGS form is important as methionine serves as a precursor for various essential metabolites. Therefore it seems reasonable that, in order to maintain methionine/SAM production at a low rate even in high methionine/SAM concentrations when the transcript level of full-length CGS has been reduced, there is an alternative deleted CGS transcript that remains expressed. Moreover, Western blot analyses performed on different Arabidopsis organs demonstrated differences in the relative intensities of the two bands representing the full-length and deleted forms of CGS proteins (Figure 5b). This suggests that these two forms of CGS are regulated at the post-translational level. We believe that the ratios between these two CGS protein forms found in Arabidopsis are one of the main factors that determine the rate of methionine synthesis in different organs.

Experimental procedures

Arabidopsis cDNA libraries

The Arabidopsis cDNA library Uni-Zap flower (CD4-6) was kindly provided by Detlef Weigel via the Arabidopsis Resource Center in Columbus, OH, USA.

RNA extraction and amplification of CGS cDNA fragments

Total RNA was extracted from Arabidopsis roots, leaves, seeds, stems and flowers using Tri-Reagent (Sigma, St Louis, MO, USA), according to the manufacturer's instructions. cDNA was synthesized using oligo(dT) primer, 1 μg of total RNA and avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA). PCR and RT-PCR analyses were performed using the method described by Frohman (1990). Fragments of DNA encoding mature CGS (without its plastid transit peptide) starting with Val-68 (Ravanel *et al.*, 1998a) were amplified using primers P1: 5′-AGCATG CTCGTCCGTCAGCT-GAGCATTAAAC (containing a *Sph*I restriction site and an ATG

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translation-initiation codon) and P2: 5′-CATAACGACCAT ACTCG AAACTC (see Figure 1d). PCR products were cloned and sequenced using an automatic sequencer (Model 373A, Applied Biosystems, Foster City, CA, USA).

Binary plasmid construction and plant transformation

The cDNAs encoding the deleted and full-length forms of CGS were PCR-amplified using the cDNA flower library and P1 primer described above and P3: 5′-ACCCGGGATGGCTTCGAGAGCTT-GAAG (containing a *Sma*I site). The PCR fragment was first introduced into the pGEMT plasmid (Promega), then digested with *Sph*I and subcloned into a pCE vector (Shaul and Galili, 1992). This vector contains the 35S promoter of cauliflower mosaic virus, an Ω DNA sequence from the coat protein gene of tobacco mosaic virus for translation enhancement, and the chloroplast transit peptide of pea rbcS-3A (Shaul and Galili, 1992). The fragment was then subcloned into the binary Ti plasmid pZP111, using *Sph*I and *Sma*I restriction sites. Using this binary vector, the DNA fragments containing CGS were fused in-frame to three copies of the hemaglutinin ($3 \times$ HA), replacing its natural stop codon. The pZP111 plasmid carries the gene for kanamycin resistance.

Tobacco plants were transformed and selected on media containing 100 mg l^{-1} kanamycin as previously described (Horsch *et al.*, 1985).

Complementing the E. coli *methionine auxotroph*

The three forms of cDNA encoding the full-length and two deleted forms of CGS were amplified from a cDNA flower library using primers 1 and 3. However, the *Sph*I restriction site was replaced by the *Bam*HI site in the forward primer, and the *Sma*I site was replaced by *Hin*dIII in the reverse primer (primer 3). *Bam*HI and *Hin*dIII were used to insert the amplified DNA fragments into the pQE30 expression vector (Qiagen, Hilden, Germany). The nucleotide sequences of the constructed plasmids were verified by DNA sequencing. The three generated plasmids were used to complement the *E. coli* methionine auxotroph mutant LE392 (*met*B1, *tryp*R55, P2 lysogen; Stratagene, La Jolla, CA, USA). The complemented mutants were cultured for 36 h at 37°C in M9 medium (Sambrook *et al.*, 1989), which was supplemented with 50 μg ml^{-1} ampicillin, tryptophan (40 mg l^{-1}) and, for the positive control, methionine (40 mg l^{-1}). The solid medium contained 1.1% agar (w/v; Sigma).

Probes for ribonuclease protection assay

To generate a vector encoding probe 421, a PCR product was obtained using primers P4: 5′-TCTAGAATGGCCGTCTCATCATTCCA-GAG and P5: 5′-GAAGAAACC GCAGATGCAGAC and cloned into pGEMT (Promega). For probe 504, the second primer was replaced with P6: 5′-CCATCGG AGCTCAAGAAAG and the deleted CGS cDNA was used as a template. Plasmids harboring the correctly oriented PCR fragment with respect to the T7 promoter were linearized with *Not*I and transcribed *in vitro* to generate the [^{32}P] antisense RNA as previously described (Meierhoff *et al.*, 2003).

Ribonuclease protection assay

Arabidopsis total RNA (20 μg) was re-suspended in 1 μl of ddH_2O and added to 14 μl of hybridization buffer (80% formamide, 400 mM NaCl, 40 mM PIPES, pH 6.4, and 1 mM EDTA) containing 1.5×10^5 cpm of gel-purified, uniformly labeled antisense RNA probe. Following heating for 10 min at 85°C, annealing was performed at 50°C for 16 h. Unhybridized RNA was digested with 120 U of RNase T1 (Sigma) in 200 μl of buffer containing 300 mM NaCl, 10 mM Tris, pH 7.4, and 5 mM EDTA for 1 h at 30°C. The reaction was stopped with the addition of stop mix (4% SDS, 0.8 mg ml^{-1} proteinase K and 150 μg ml^{-1} tRNA) and incubated at 37°C for 15 min followed by phenol–chloroform extraction and ethanol precipitation. For size markers, *in vitro* generated transcripts of known sizes were used.

Western blot analysis

Leaves of transgenic and wild-type plants were homogenized as previously described (Hacham *et al.*, 2002). Protein samples (10–30 μg) were fractionated on 10% SDS-PAGE and transferred to a cellulose nitrate membrane using Bio-Rad Protein Trans-Blot apparatus (Bio-Rad, Hercules, CA, USA). The membrane was blocked overnight at 4°C in a solution of 5% (v/v) non-fat dried milk, and then reacted with commercial anti-HA monoclonal antibodies (Roche, Mannheim, Germany) or CGS rabbit antiserum for 2 h at room temperature. Immunodetection was conducted with an enhanced chemiluminescence kit (Pierce, Rockford, IL, USA) in accordance with the manufacturer's instructions.

Polysome fractionation

Polysome analysis on sucrose gradients was performed as described before (Barkan, 1989; Rott *et al.*, 1999). RNA was purified from the fractions indicated and subjected to ribonuclease protection analysis as described above. In control samples, polysomes were dissociated by the addition of EDTA (20 mM) to the plant lysates prior to gradient loading. In these gradients, 1 mM EDTA was substituted for 10 mM $MgCl_2$ in the isolation buffer.

Analysis of amino acids

The transgenic tobacco plants expressing the deleted version of CGS were analyzed for free amino acids as previously described (Hacham *et al.*, 2002). Briefly, free amino acids were extracted from frozen leaves of 8-week-old plants and the amino acid composition was determined by loading 66 nmol on a Hewlett-Packard Amino Quant Liquid Chromatograph (Agilent Technologie, Waldbronn, Germany).

RNA structure analysis

The secondary mRNA structure was predicted using the programs mfold and Hewlett-Vienna RNA package (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/form1.cgi; http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi).

Feeding experiments using an in vitro *transcription/translation system*

The two cDNA forms of CGS were ligated into the pBlueScript KS$^-$ (Stratagene) containing the 3HA epitope tag via *Sac*II and *Sma*I restrictions sites and they were subjected to further analysis. *In vitro* transcription and translation of the full-length and deleted forms of CGS cDNA were performed using the TNT coupled wheat germ system (Promega). Reaction mixtures (25 μl each) were prepared by utilizing an amino acid mixture lacking methionine supplied by the manufacturer. Final concentrations of 1 and 5 mM methionine were

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adjusted by adding appropriate quantities of amino acid separately to each reaction mixture. As a control, respective cDNAs were expressed in the presence of 4 μM methionine. Following incubation at 30°C for 90 min, the reactions were stopped by freezing the samples in liquid nitrogen. The CGS protein levels were subsequently determined via Western blot analysis as described above.

Acknowledgements

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The accession number of the 90-pb deleted form is AY545074

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