Identification of a Short Highly Conserved Amino Acid Sequence as the Functional Region Required for Posttranscriptional Autoregulation of the Cystathionine γ-Synthase Gene in Arabidopsis*

Cystathionine γ-synthase (CGS) catalyzes the first committed step of Met biosynthesis in plants. We have previously shown that expression of the gene for CGS is feedback-regulated at the level of mRNA stability, and that the amino acid sequence encoded by the first exon of the CGS gene itself is responsible for the regulation (Chiba, Y., Ishikawa, M., Kijima, F., Tyson, R. H., Kim, J., Yamamoto, A., Nambara, E., Leustek, T., Walls, R. M., and Naito, S. (1999) Science 286, 1371–1374). To identify the functional region within CGS exon 1, deletion analysis was performed. The results showed that the 41-amino acid region of exon 1 highly conserved among plant species is necessary and sufficient for the regulation. Analyses of in vivo and in vitro generated mutations that abolish the regulation identified the functionally important amino acids as 11–13 residues within this conserved region. The importance of these residues was confirmed by deletion analysis within the conserved region. These studies identified the functional region of CGS exon 1 required for the posttranscriptional autoregulation of the CGS gene as a small region that is highly conserved among four plant species (10, 11).

In higher plants, the first committed step of Met biosynthesis is represented by the condensation of O-phosphohomoserine and cysteine to form cystathionine (1). This reaction is catalyzed by cystathionine γ-synthase (CGS; EC 4.2.99.9) and has been proposed to be the key regulatory step in the biosynthesis pathway (2–4).

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Characterization of mto1 Mutants—Analysis of ethionine resistance and measurement of soluble Met content were carried out as described (5, 12). Extraction of total RNA from rosette leaves and Northern blot analysis were performed as described (5).

Functional Amino Acid Residues for Autoregulation of CGS

**Table I. Mutations used in this study**

| Name  | Amino acid change | Nucleotide change* |
|-------|-------------------|--------------------|
| mto1 mutations |                     |                    |
| mto1-1  | Gly-84 → Ser      | GGT → AGT          |
| mto1-2  | Ser-81 → Asn      | AGC → AAC          |
| mto1-3,5 | Gly-84 → Asp      | GGT → GAT          |
| mto1-4  | Arg-77 → His      | CGT → CAT          |
| mto1-6  | Ala-86 → Val      | GCA → GTA          |
| mto1-7  | Arg-78 → Lys      | AGA → AAA          |

| Ala substitution mutations |                     |                    |
|---------------------------|---------------------|--------------------|
| I61A-1                    | Ile-61 → Ala       | ATC → GCC          |
| P65A-1                    | Pro-65 → Ala       | CCT → GTT          |
| F68A-1                    | Phe-68 → Ala       | TTC → GCC          |
| V69A-1                    | Val-69 → Ala       | GCT → GCT          |
| R70A-1                    | Arg-70 → Ala       | CGT → GCT          |
| S73A-1                    | Ser-73 → Ala       | AGC → GCC          |
| K75A-1                    | Lys-75 → Ala       | AAA → GCA          |
| R77A-1                    | Arg-77 → Ala       | CGT → GCT          |
| N79A-1                    | Asn-79 → Ala       | AAC → GGC          |
| S81A-1                    | Ser-81 → Ala       | AGC → GCC          |
| I83A-1                    | Ile-83 → Ala       | ACT → CTC          |
| Q87A-1                    | Glu-87 → Ala       | CAG → CGG          |
| V89A-1                    | Val-89 → Ala       | GTG → CGC          |
| W93A-1                    | Trp-93 → Ala       | TGG → CGG          |

| Ser-81 site mutations |                     |                    |
|-----------------------|---------------------|--------------------|
| S81G-1                 | Ser-81 → Gly       | AGC → GGA          |
| S81C-1                 | Ser-81 → Cys       | AGC → GTC          |
| S81Q-1                 | Ser-81 → Gln       | AGC → GAT          |
| S81M-1                 | Ser-81 → Met       | AGC → ATG          |
| S81T-1                 | Ser-81 → Thr       | AGC → ACT          |
| S81V-1                 | Ser-81 → Val       | AGC → GTC          |
| S81L-1                 | Ser-81 → Leu       | AGC → CTT          |
| S81H-1                 | Ser-81 → His       | AGC → CAT          |
| S81K-1                 | Ser-81 → Lys       | AGC → AGA          |
| S81R-1                 | Ser-81 → Arg       | AGC → AGA          |
| S81D-1                 | Ser-81 → Asp       | AGC → GAT          |
| S81E-1                 | Ser-81 → Glu       | AGC → GAG          |
| S81F-1                 | Ser-81 → Phe       | AGC → AGG          |
| S81P-1                 | Ser-81 → Pro       | AGC → CCT          |

* Mutated bases are underlined.

based on a phenotype of resistance to ethionine, a toxic analog of Met (15). Genetic mapping and sequence analysis were carried out as described (6). The mto1-6 and mto1-7 mutations thus identified were used for further study after being backcrossed three times to wild-type plants. Plant growth conditions have been described (14, 15).

**Characterization of mto1 Mutants—Analysis of ethionine resistance and measurement of soluble Met content were carried out as described** (5, 12). Extraction of total RNA from rosette leaves and Northern blot analysis were performed as described (5).

**Plasmid Construction for Transient Expression Experiments—**Full-length CGS exon 1 (amino acids 1–183) constructs that contain either full-length CGS exon 1 sequences fused in-frame with Escherichia coli β-glucuronidase (GUS) reporter gene and placed under the control of the CaMV 35 S RNA promoter have been described (6). The wild-type and mto1-6 mutant versions of this plasmid are referred to as pM14(WT) and pM14(mto1-6), respectively. For the deletion and reverse primers Ex1P1 (5′-CGAATCTAGAATGGCCGTCTC-3′) and Ex1P2 (5′-ATCTAGATCCACCGGATG-3′) that were used to amplify the exon 1 sequences by polymerase chain reaction (PCR) carry XbaI and BamHI recognition sequences (underlined), respectively (6).

The deletion series of CGS exon 1 was constructed by PCR amplification of the respective regions using pM14(WT) as a template. For the C-terminal deletions, the forward primer was Ex1P1, and the reverse primers were C1r (5′-GAAGACGGATCCATCTGATGATC-3′), C2r (5′-ATCGATCTAATACACAGCTG-3′), C3r (5′-AAGAATCCGGATGATTGCTG-3′), and C4r (5′-ACGAGCCGATCCGGCCGACGCGACG-3′) for CD1 (deletion of amino acids 28–183), CD2 (amino acids 58–183), CD3 (amino acids 99–183), and CD4 (amino acids 137–183), respectively. The forward and reverse primers Ex1P1 (5′-CGAATCTAGAATGGCCGTCTC-3′) and Ex1P2 (5′-ATCTAGATCCACCGGATG-3′) that were used to amplify the exon 1 sequences by polymerase chain reaction (PCR) carry XbaI and BamHI recognition sequences (underlined), respectively (6).

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**Ethionine resistance, soluble Met concentration, and CGS mRNA accumulation in mto1 mutant alleles.** A, wild-type (WT) and alleles of mto1 mutant seeds were sown on agar-solidified plates containing various concentrations of L-ethionine as indicated, and photographs were taken after 14 days of incubation. B, amino acids were extracted from rosette leaves 21 days after imbibition, and soluble Met concentration was determined. Soluble Met concentrations relative to that in wild type are also indicated. C, total RNA was extracted from rosette leaves at 21 days after imbibition, and CGS mRNA accumulation was determined by Northern hybridization using CGS cDNA (29) as a probe (upper panel). The membrane was rehybridized with a ubiquitin cDNA (30) probe (UBQ) as a loading control (lower panel).
For the deletion constructs retaining the mto1-1 mutation site, namely CD3, CD4, ND5, ND6, IR1, IR2, IR3, IR4, and dL1, mto1-1 mutant versions of each construct were generated using pMI4(mto1-1) as the template for PCR amplification. Ala substitution mutagenesis (17) and amino acid changes at the Ser-81 position (Table I) were carried out by the overlap extension PCR method (18, 19). The flanking primers were Ex1P1 and Ex1P2. Sequences of the 62 mutagenic internal primers will be provided upon request. The mutant CGS exon 1 sequences were cloned into pHAI between the XhoI and BamHI sites. pHAI is a pUC19 vector that carries the CaMV 35 S RNA promoter:CGS exon 1:GUS:nopaline synthase terminator fragment from PMI4(WT) between the HindIII and EcoRI sites. In all cases, the integrity of PCR-amplified regions was confirmed by sequence analysis.

The control construct ΔS–183 that carries only the first four amino acids of CGS exon 1 fused in-frame to the GUS reporter gene and placed under the control of CaMV 35 S RNA promoter has been described (6). The 221-LUC+ plasmid carries a modified firefly luciferase gene directly under the CaMV 35 S RNA promoter (20) and was used as an internal standard for transient expression experiments.

Transient Expression Studies—Liquid callus cultures of Arabidopsis were prepared as described (21, 22), and transfection of tester (GUS reporter) and control (luciferase reporter) plasmids by electroporation was carried out as described (6).

Identification and Characterization of mto1 Alleles—We have previously shown that the coding region of the first exon of CGS (183 amino acids) is sufficient for the regulation of its own mRNA stability (6, 7). Although the amino acid sequences and lengths of CGS exon 1 region are not well conserved, there was a region within exon 1 that is highly conserved among four plant species (6). In the present study, the region between amino acids 58 and 98 of CGS exon 1 was referred to as the conserved region. The observation that the three amino acids altered by previously identified mto1 mutations (mto1-1 to mto1-5) (Table I) were clustered in the conserved region (6) implies an important role for this conserved region in the posttranscriptional regulation of the CGS gene. To identify the functionally important region in CGS exon 1, we isolated additional mto1 alleles. Two new mutations were identified and designated as mto1-6 and mto1-7, respectively (Table I). These mutations are also located within the conserved region in close proximity to the previously identified mto1 mutations (6).

The seven mto1 alleles we have so far isolated were characterized on the basis of ethionine resistance, soluble Met accumulation, and CGS mRNA accumulation. As shown in Fig. 1A, growth of wild-type plants was severely inhibited by 30 μM of L-ethionine (5), whereas growth of all seven mto1 mutants was

Fig. 2. Transient expression studies of deletion constructs. A, schematic diagram of deletion constructs. The CGS exon 1 region retained by each construct is shown. Black bars indicate the conserved region. The position of the mto1-1 mutation is also indicated. Exon 1, the full-length exon 1 construct. B, deletion constructs derived from pMI4(WT) were transfected to wild-type Arabidopsis protoplasts and incubated for 48 h in the presence and absence of 0.1 mM Met. GUS activity was first normalized with co-transfected luciferase activity, and the ratio of Met-treated to nontreated was calculated. Averages ± S.D. of at least triplicate experiments are shown. C, deletion constructs derived from pMI4(mto1-1) were analyzed as in B.
not affected at this concentration of L-ethionine. mto1-4 and mto1-6 mutants showed a weaker resistance to ethionine than the other alleles, and their growth was inhibited in the presence of 100–200 μM L-ethionine.

Soluble Met concentrations in rosette leaves of mto1-1, -2, -3, -5, and -7 were 30–40-fold higher than wild-type (Fig. 1B). On the other hand, mto1-4 and mto1-6 accumulated lower concentrations of soluble Met than the other alleles, consistent with its weak ethionine-resistant phenotype.

CGS mRNA accumulated to higher levels than wild-type plants in all mto1 mutant alleles (Fig. 1C). The weak mto1-4 and mto1-6 alleles showed lower levels of CGS mRNA accumulation compared with that in the other alleles. These results demonstrate a positive correlation between CGS mRNA accumulation level, soluble Met accumulation level, and ethionine resistance. CGS protein accumulation levels were also similar to that for CGS mRNA accumulation.

Although the mto1-3 and mto1-5 mutants were isolated independently, they carry the same mutation (6). Consistent with this, these two mutants exhibited similar phenotypes (Fig. 1; data not shown).

**Deletion Analysis of the CGS Exon 1**—To test whether the conserved region (amino acids 58–98) is necessary for the ability to down-regulate its own expression in response to Met application, N- and C-terminal deletions of CGS exon 1 were constructed and transient expression studies were carried out (Fig. 2). Expression of the deletion constructs that lacked the conserved region (CD1, CD2, ND7, and ND8; Fig. 2A) was not down-regulated in response to applied Met (Fig. 2B), indicating that the conserved region is necessary for the regulation. In contrast, deletion constructs that retained the conserved region (CD3, CD4, ND5, and ND6; Fig. 2A) did respond to applied Met. Although the level of response was weaker than that for the full-length CGS exon 1 construct (Fig. 2A, Exon 1), the positive response to applied Met was substantiated by the observation that introduction of mto1-1 mutation into these constructs totally abolished the response (Fig. 2C).

To test whether the conserved region is sufficient for the regulation, constructs bearing both N- and C-terminal deletions (IR1, IR2, IR3, and IR4; Fig. 2A) were also generated and tested (Fig. 2B). The results showed that all of these constructs responded to applied Met even when the conserved region alone was used (IR3). Although again the response was weaker than that for the full-length exon 1 construct, introduction of mto1-1 mutation totally abolished the response (Fig. 2C). The results indicate that the conserved region is essentially sufficient for the regulation.

**Mutational Analyses of the Conserved Region in the CGS**

Figure 4. Effect of amino acid changes at the Ser-81 position in transient expression system. Full-length exon 1 constructs carrying wild type (Ser), mto1-2 (Asn), and all other 18 protein amino acids as indicated were transfected to wild-type Arabidopsis protoplasts, and response of the GUS activity to applied Met was analyzed as in Fig. 2B. Averages ± S.D. of at least triplicate experiments are shown. Asterisks indicate significant difference (p < 0.05 by t test) from the full-length wild-type exon 1 construct (WT).

Exon 1—The full-length exon 1 constructs carrying mto1-6 and mto1-7 mutations were generated, and the response to applied Met was tested (Fig. 3). Constructs carrying mto1-1 to mto1-4 mutations that we have previously reported (6) were reanalyzed for comparison. As expected, both mto1-6 and mto1-7 mutations affected the response, as did the other mto1 alleles.

In order to determine which amino acids of the conserved region in CGS exon 1 are important for the regulation, effects of alanine substitution mutations (17) were studied in the context of full-length exon 1. Of the 41 amino acids in the conserved region, 14 amino acids were individually changed to alanine, and their response to applied Met was tested (Fig. 3). As a result, four additional mutations were identified, namely R77A-1, N79A-1, I83A-1, and Q87A-1, whose changes impaired the response. The change of Ser-81 to alanine in SS1A-1, however, did not impair the regulation. This was despite the fact that the regulation was abolished by alteration of the same amino acid residue to asparagine in the mto1-2 mutant. Due to this unexpected result, Ser-81 was changed to all other amino acids and tested further (Fig. 4). The results showed that amino acids with a small side chain, namely serine (wild type), glycine, alanine, and cysteine, were tolerated, whereas changes to those with a larger side chain affected the regulation.

Critical amino acids for the regulation identified by alanine substitution mutagenesis were clustered in a small region where all of the mto1 mutations are also located (amino acids 77–87) (Fig. 3). This implied that this 11-amino acid sequence within the conserved region is essential for the regulation of the CGS gene. To confirm this, additional deletion constructs within the conserved region were generated and tested. As expected, deletion of amino acids 77–87 in the dR1 construct (Fig. 2A) abolished the response to applied Met. In contrast, deletion of amino acids 61–68 in the dL1 construct (Fig. 2A), a C-terminal part of the conserved region, still showed a response (Fig. 2B). The positive response of the dL1 construct was evidenced by introduction of the mto1-1 mutation, which totally abolished the response (Fig. 2C).

**DISCUSSION**

The results presented in this study identified the region of CGS exon 1 that is critical for the posttranscriptional regulation of its own expression in response to applied Met. We have previously denoted the amino acid sequence defined by mto1 mutations as the MTO1 region (6). The results obtained in this study demonstrated that the MTO1 region spans from amino acid 77 to 87 (Arg–Arg–Aan–Cys–Ser–Aan–Ile–Gly–Val–Aal–Gln). Untested Ala-76 and Ile-88 might also be included in the MTO1 region. Changes of amino acids near the border of the MTO1 region.
Fig. 5. Alignment of nucleotide and amino acid sequences of a CGS region from various plant species. A, alignment of amino acid sequence corresponding to the Arabidopsis CGS exon 1 region. BLASTp searches (26) of the protein data base was carried out using the exon 1 region of Arabidopsis Col-0 ecotype (accession number AF039206) as the query. BLASTn searches of the expressed sequence tag data base were also carried out using the exon 1 region of Arabidopsis Col-0 ecotype and the corresponding region of Zea mays (accession number AF067785) as the query. Protein data base entries that were identified only by genome sequencing were not chosen. For those sequences with multiple expressed sequence tag entries, a consensus sequence was deduced by comparing the nucleotide alignment. The deduced amino acid sequences were aligned with the ClustalW program (31) (available on the World Wide Web at clustalw.genome.ad.jp/).

Dashes indicate gaps in the alignment, and filled circles indicate sequences that are missing in the database. Identical (asterisks) and similar (dots and colons) amino acids were marked both for the angiosperm sequences (ang) and for all of the sequences (all). Shaded and nonshaded sequences indicate species of the same family.

The horizontal bar above the alignment indicates the MTO1 region. Deletion ends of CD and ND series and the exon 1/2 junction in Arabidopsis are also indicated.

AtC, Arabidopsis Col-0 ecotype; AtL, Arabidopsis Ler ecotype; Hv, Hordeum vulgare (barley); Ta, Triticum aestivum (bread wheat); Zm, Zea mays (maize); Sp, Sorghum bicolor; GaA, Gossypium arboreum (tree cotton); GmA, Glycine max (soybean); Lj, Lotus japonicus; Mt, Medicago truncatula (barrel medic); Le, Lycopersicon esculentum (tomato); Lp, Lycopersicon pennellii (wild tomato); StA, Solanum tuberosum (potato); Nt, Nicotiana tabacum (common tobacco); Mc, Mesembryanthemum crystallinum (common ice plant); PpA, Physcomitrella patens.

B, alignment of nucleotide sequence corresponding to amino acids 61–95 of Arabidopsis CGS. The sequences are from the same source as in A. Missing sequences are marked with filled circles. Identical nucleotides among the angiosperm sequences (ang) and all of the sequences (all) are marked with asterisks.
region (Arg-77, Ala-86, and Gln-87) had a weaker effect on the regulation compared with that in the central part of the MTO1 region (Fig. 3). Interestingly, the weak alleles of mto1-4 and mto1-6 are among those near the border.

Detailed analysis of the Ser-81 position showed that those amino acids with a small side chain are tolerated. Other characteristics such as polar/nonpolar did not seem to affect the regulation (Fig. 4). The most likely interpretation of this result is that amino acids with a larger side chain inhibit the function of the MTO1 region by a structural hindrance at this position. The same rule, however, does not seem to apply for Gly-84, because the mto1-1 mutation that alters this amino acid to serine strongly abolished the regulation.

Due to the fact that not all amino acids in the conserved region were tested by alanine substitution mutagenesis and that deletion analysis within the conserved region did not cover all residues, we cannot rule out the possibility that amino acids outside the MTO1 region are also involved in the regulation. No mto1 mutation, however, has been identified outside the MTO1 region from our screens. This is despite the fact that mutations were independently found three times at the same amino acid residue in the MTO1 region (Gly-84 for mto1-1, -3, and -5) (6). Ethyl methanesulfonate, the mutagen used for mutation, however, has been identified outside the MTO1 region (Fig. 5A). On the other hand, tBLASTn searches (26, 27) of the GenBank™ databases did not identify any sequence that has the RRNC-SNIG(V/L)AQ sequence other than those for plant CGS sequences.

Expressed sequence tags of Chlamydomonas reinhardtii (accession numbers BF862446, BI874449, and BF866840) that were identified in the tBLASTn searches showed significant (expect value <10−30) homology to the Arabidopsis CGS exon 2 plus 3 region. They also encode a 75-amino acid N-terminal extension sequence but did not contain the MTO1 sequence (data not shown), suggesting that the MTO1 sequence is unique to CGS of multicellular plants.

In contrast to the highly conserved nature of the amino acid sequences in the MTO1 region, nucleotide sequences encoding this region carry many synonymous changes (Fig. 5B). The present observation supports our previous conclusion that it is the amino acid sequence and not the nucleotide sequence that has a role in the regulation (6).

A Prosite data base search (28) (available on the World Wide Web at www.expasy.ch/tools/scanprosite/) of the MTO1 region identified only an asparagine N-glycosylation site (Asn-Cys-Ser-Asn). However, N-glycosylation does not seem to be relevant to the function of a nascent polypeptide. Although there is still much to be understood regarding regulation of the CGS gene in response to applied Met, the functional amino acid sequence identified in this study provides a critical key to the elucidation of this unique regulatory mechanism.

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Identification of a Short Highly Conserved Amino Acid Sequence as the Functional Region Required for Posttranscriptional Autoregulation of the Cystathionine \( \gamma \) -Synthase Gene in \textit{Arabidopsis}

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