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Regulation of Methionine Biosynthesis in Plants; Transgenic Study

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식물에서 메티오닌 생합성의 조절; 형질전환체 연구

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ABSTRACT : The committing step in Met and *S*-adenosyl-L-methionine (SAM) synthesis is catalyzed by cystathionine γ-synthase (CGS). Transgenic *Arabidopsis thaliana* overexpressing CGS under control of 35S promoter show increased soluble Met and its metabolite *S*-methylmethionine, but only at specific stages of development. CGS-overexpressing seedlings are resistant to ethionine. Similar results were obtained with transgenic potato plants overexpressing Arabidopsis CGS. Several of the transgenic lines show silencing of CGS resulting in deformed plants with a reduced capacity for reproductive growth similar as transgenic plants by antisense RNA (CGS[-]). Exogenous feeding of Met to the CGS[-] and CGS[+] silenced plants partially restores their growth. Similar morphological deformities are observed in plants cosuppressed for SAM synthetase, even though such plants accumulate 250 fold more soluble Met than wild type and they overexpress CGS. The results suggest that the abnormalities associated with CGS and SAM synthetase silencing are due in part to a reduced ability to produce SAM, and that SAM may be a regulator of CGS expression.

Key words : methionine biosynthesis, cystathionine γ-synthase, threonine synthase, S-adenosylmethionine, *Arabidopsis thaliana*.

INTRODUCTION

Met is derived from Asp as are the amino acids Lys, Thr, and Ile. The committing step in Met synthesis occurs when the side chain of *O*-phosphohomoserine (OPH) condenses with the thiol group of Cys to form cystathionine (Fig 1), an irreversible reaction catalyzed by cystathionine γ-synthase (CGS)(EC 4.2.99.9). Cystathionine is cleaved to form homocysteine, which is then methylated with tetrahydrofolate (THF) to form Met. The major metabolic fates of Met include its incorporation into protein, adenosylation to form *S*-adenosylmethionine (SAM), and methylation to

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form *S*-methylmethionine (SMM) (Fig. 1).

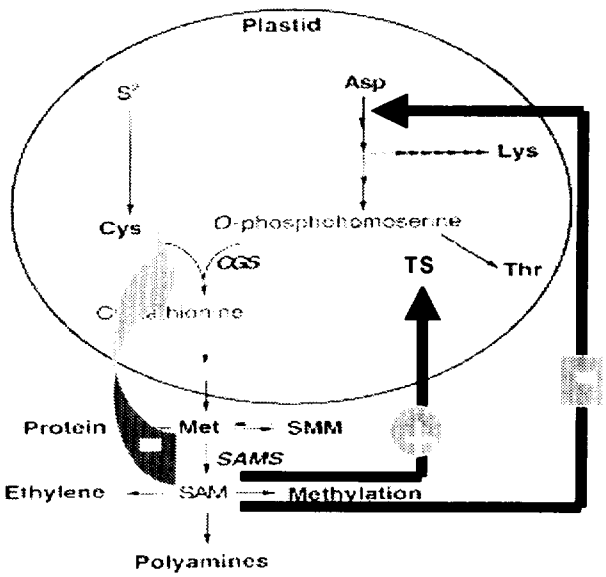

Fig. 1. Pathway for synthesis of Met, SAM and Thr. The pathway is as described in the text. SMM, *S*-methylmethionine; SAM, S-adenosyl-L-methionine; CGS, cystathionine γ -synthase; TS, threonine synthase. Solid arrows refer to enzymatic steps and bold arrows refer to regulatory steps.

CGS competes with threonine synthase (TS) for OPH, their common substrate. Thus, TS may exert some control over the rate with which OPH is channeled toward Met (Bartlem et al., 2000; Fig. 1). TS is allostrically regulated by SAM (Curien et al., 1998) suggesting that Met synthesis could influence TS activity. Even so, several lines of evidence indicate that CGS controls the rate of Met synthesis. CGS activity decreases when Met is fed to the aquatic angiosperm *Lemna* and increases when Met synthesis is blocked by inhibiting aspartokinase, the first enzyme in the biosynthesis of the Asp family of amino acids (Thompson et al., 1982). In the *Arabidopsis thaliana* mutant *mto1*, CGS is overexpressed, resulting in overaccumulation of soluble Met (Chiba et al., 1999, Inaba et al., 1994). Finally, antisense-RNA repression of CGS expression results in growth deformities stemming from an inability to synthesize Met (Kim and Leustek, 2000).

CGS expression may be regulated in Arabidopsis by an autogenous mechanism, revealed through analysis of the mutant *mto1* (Chiba et al., 1999). In wild type Arabidopsis, Met or a metabolite thereof, down-regulates CGS enzyme expression (Fig 1) through a post-translational mechanism that acts by destabilizing CGS mRNA. In *mto1*, a point mutation in exon 1 of the CGS gene abolishes the Met-dependent destabilization of CGS mRNA causing the enzyme level and soluble Met level to rise. Both *mto1* was isolated by selection for Arabidopsis mutants that are resistant to ethionine, a toxic Met analog. Ethionine-resistance arises from overaccumulation of soluble Met.

In this study a transgenic approach was used to study the role that CGS protein abundance plays in controlling the level of free Met in Arabidopsis. The results show that transcriptional upregulation of CGS causes accumulation of soluble Met and SMM, but only in specific tissues and stages of development. The results also show that transcriptional upregulation of CGS can overcome the post-transcriptional mechanism controlling CGS expression. Cosuppression of CGS causes pronounced morphological aberrations and physiological changes that resemble those observed in Arabidopsis plants in which SAMS is cosuppressed. Comparative analysis of CGS and SAMS silenced plants suggests that SAM may be a regulator of CGS expression and that SAM deficiency may cause the morphological and physiological aberrations.

RESULTS

Isolation and initial characterization of Arabidopsis lines with altered expression of CGS

Transgenic Arabidopsis plants were isolated from transformations with a construct intended to produce stable overexpression of CGS. A representative blot of leaf tissue from primary transformants analyzed for CGS protein level by immunoblotting shows that transgenic plants were isolated with a diversity of CGS levels in leaf tissue ranging from high-level expression to plants from which CGS protein could not be detected (Fig. 2). Two transgenic lines were chosen for further analysis, each from an independently transformed plant. RNA and immunoblot analysis showed that CGS is overexpressed in the leaves of 27 d old plants.

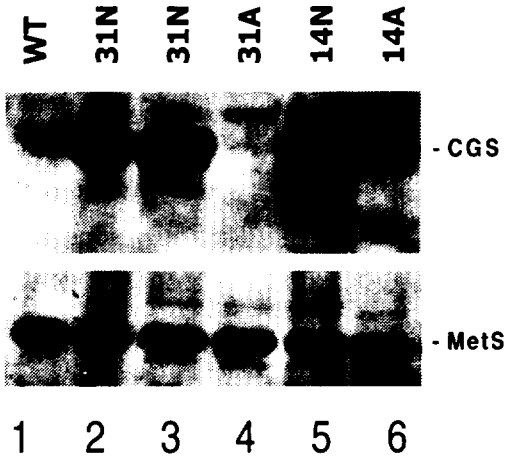

Fig. 2. Immunoblot analysis of CGS expression. Homozygous CGS transgenic plants were grown in potting mix for 30-d. Ten micrograms of leaf protein from normal (N) and abnormal (A) plants were analyzed. Immunoblots were reacted with antiserum against CGS. MetS serves as a protein loading control.

Plants that overexpress CGS are resistant to ethionine

Plants that overexpress CGS are phenotypically normal compared with wild type. Ethionine resistance for two of them, #14 and #31 are shown in Fig. 3 compared with *mto1*. Ethionine is a toxic Met analog and resistance can be overcome by overproduction of soluble Met (Inaba et al., 1994; Bartlem et al., 2000). This result indicates that CGS overexpression has probably resulted in Met overproduction in Arabidopsis seedlings.

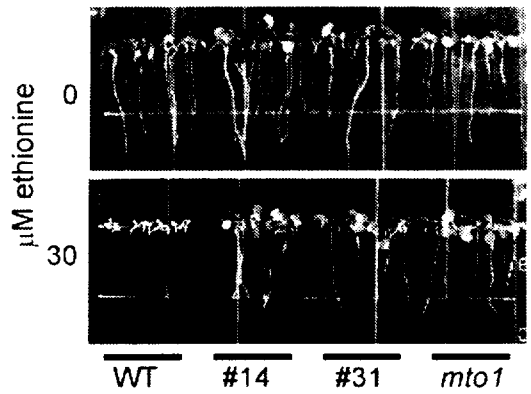

Fig. 3. Resistance of CGS-overexpressing plants to ethionine. Ethionine resistance were performed on homozygous progeny of the indicated primary transgenic plants. The plants were grown on the indicated ethionine concentration.

Plants that overexpress CGS accumulate free Met and SMM

Analysis of soluble amino acids revealed that Met and SMM accumulate in the CGS-overexpressing plants (Fig. 4), but their level is strongly dependent on the developmental stage and organ. The highest levels occur in the leaf and root of seedlings and in the root and flowers of mature plants. The accumulation of soluble SMM is more pronounced than is the accumulation of Met. The high accumulation of soluble Met and SMM in seedlings correlates with the finding that seedlings are resistant to ethionine (Fig. 3). As the CGS-overexpressing transgenic plants begin to flower the level of Met and SMM declines in leaf tissue. The level of these metabolites in roots is maintained, although the variation between plant samples increases. As the plants progress through the flowering stage of development the level of Met and SMM in the leaf

of transgenic plants declines further and is nearly indistinguishable from wild type, whereas the level remains high in flowers.

Tubers and roots in transgenic potato overexpressing CGS accumulate free Met

Met is the precursor of the primary flavor compound in potato, methional, which is synthesized from Met by the Strecker degradation reaction. In order to enhance free Met levels in potato, the *Arabidopsis thaliana* CGS cDNA was introduced into Russet Burbank potato plants by *Agrobacterium*-mediated transformation. Ten different transgenic potato lines (CGS1-10) were generated. Southern blot analysis revealed that five lines are independent transformants. Immunoblot analysis demonstrated that CGS is overexpressed in leaves, tubers and roots of transgenic plants, with CGS1 and CGS8 as the highest producers. Although the CGS enzyme activity was 2-7 fold higher in the leaves of five independent transgenic lines, Met levels in the leaves were similar to the levels in wild type potato leaves. In contrast, Met levels in the tubers and roots of transgenic plants were 2 to 5-fold higher than the levels in wild type, with CGS1 as the highest (Kim, 2000). All transgenic lines were resistant to ethionine (Tumer, unpublished results). These results demonstrated that overexpression of a heterologous CGS enzyme, increases Met levels in potato tubers and roots, but not in leaves, suggesting that soluble Met may be translocated from the vegetative organs to the sink organs in potato.

Silencing of CGS is associated with growth and metabolic abnormalities

Five of the primary transgenic plants derived from transformation with the construct intended for CGS overexpression did not show immunodetectable CGS protein (Fig. 2). The growth of these individuals was severely stunted and they were unable to produce inflorescences. The plants developed many, very small leaves, resulting from proliferation of numerous apical shoots, an indication that they may have reduced apical dominance. Application of Met as an addition to the watering solution stimulated vegetative and reproductive growth, although a completely normal morphology was not restored. However, with Met feeding the plants were able to set viable seeds, which they could not without Met feeding. The seeds from Met-rescued plants in the second and later generations produced both normal and abnormal progeny indicating that CGS silencing is unstable. The primary transgenic plants that initially showed CGS-overexpression also produced growth-stunted progeny that could be rescued by Met-feeding. The simplest explanation for the phenotype of these plants is that silencing of CGS expression resulted in an inability to synthesize sufficient Met, or a metabolite thereof, for growth and that CGS silencing occurs spontaneously in response to epigenetic factors.

The morphology of CGS-silenced plants resembles that associated with SAM synthetase silencing

During the course of this study it became evident that the morphology resulting from CGS silencing is remarkably similar to that of transgenic Arabidopsis cosuppressed for SAM synthetase (SAMS). Preliminary characterization of SAMS silencing in Arabidopsis has been reported by de Carvalho et al. (1994). Transgenic Arabidopsis lines showing cosuppression for CGS and SAMS were grown side by side for a more detailed comparison. The phenomena of CGS and SAMS cosuppression share the properties of

being sporadic, hyper-variable, and localized to sectors on a single plant. The plants in which SAMS becomes cosuppressed early in development are severely stunted, produce numerous apical shoots, and are unable to flower. These are termed the MUT3 morphotype using the nomenclature of de Carvalho et al. (1994). MUT3 plants are nearly indistinguishable from plants in which CGS becomes cosuppressed early in development. A major difference is that the growth of SAMS cosuppressed plants cannot be restored by exogenous application of Met. Plants that develop SAMS cosuppression later in development produce curled, chlorotic leaves, and distorted siliques resulting from early enlargement of ovules, similar to plants where CGS becomes silenced later in development. These are termed the MUT2 morphotype (de Carvalho et al., 1994). Plants that overexpress SAMS are morphologically indistinguishable from wild type and are termed the MUT1 morphotype (de Carvalho et al., 1994).

Silencing of SAMS causes CGS overexpression and accumulation of Met, SMM and other amino acids

In considering the significant morphological similarities associated with SAMS and CGS silencing it is noteworthy that these enzymes are metabolic neighbors in the pathway for SAM synthesis. It was, therefore, of interest to compare the physiological properties of SAMS silenced plants with those that overexpress SAMS and wild type. Immunoblot analysis confirmed previously that the SAMS protein is overproduced in the leaves of the MUT1 morphotype and is reduced in the MUT2 and MUT3 morphotypes (Fig. 5), as has previously been shown by SAMS activity measurements (de Carvalho et al., 1994). In contrast, CGS is overexpressed in MUT2 and MUT3, while its expression is slightly lower in MUT1 compared to wild type. Measurement of CGS enzyme activity in the leaves of MUT3 plants revealed that it is 0.91 ±0.03 nmol min-1 mg protein-1, compared with wild type activity (0.33 ±0.06) indicating that MUT3 plants have approximately 3 fold higher CGS activity than do wild type plants. In contrast, SAT expression is unaffected.

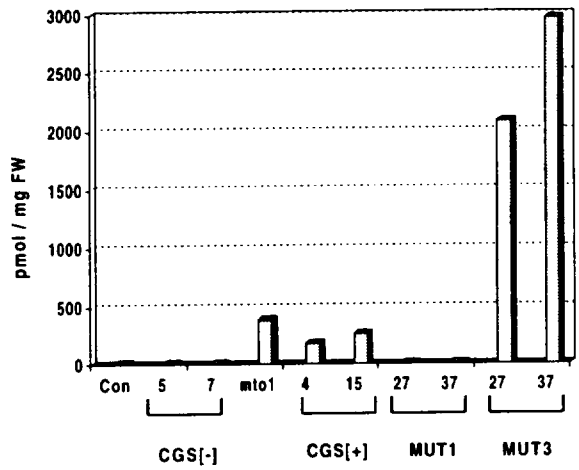

Fig. 4. Soluble Met levels in tissues of wild type and transgenic plants. Cauline leaves were harvested from 12 d plants grown in potting mix.

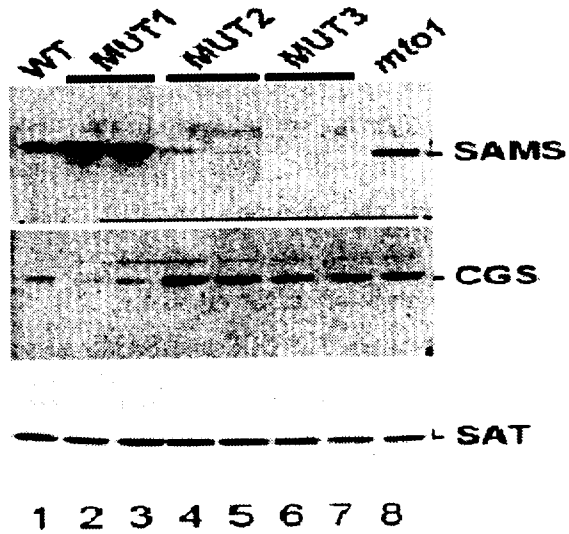

Fig. 5. Immunoblot analysis of leaf tissue from plants transformed with the SAMS construct. The plants were grown for 35 d in potting mix and leaf samples from two individual plants of each morphotype were analyzed along with wild-type and the *mto1* mutant.

Amino acid analysis revealed that the level of soluble Met and SMM are similar in wild type and in MUT1, but these metabolites accumulate to high levels in MUT2 and MUT3 (Fig. 4). This property is in contrast to CGS[-] plants, which do not accumulate Met and SMM. However, Thr and total amino acids are markedly increased.

DISCUSSION

The rationale for overexpressing CGS and SAMS in transgenic Arabidopsis was to test whether these enzymes plays a role in regulation of Met synthesis. The accumulation of soluble Met and SMM in the transgenic lines overexpressing CGS indicates that CGS is indeed a control point. The evidence on CGS transgenics, in combination with analysis of SAMS transgenic plants, produced insight into 4 additional points. (1) The observation that Met accumulation does not correlate with CGS level in a specific tissue indicates that the tissue concentration of soluble Met is controlled by factors other than the level of CGS activity. (2) The observation that CGS is not overproduced in some plant tissues, even though expression was transcriptionally deregulated from the transgene construct, suggests that a post- transcriptional mechanism may be involved in control of CGS expression. (3) The morphological similarity of CGS and SAMS silenced plants suggest that the growth abnormalities are due in part to a reduced ability to produce SAM. (4) The observation that CGS is overexpressed in SAMS silenced plants provides indirect evidence that CGS expression may be controlled by SAM or some metabolite downstream of SAM.

The leading hypothesis for the control of Met synthesis proposes that regulation occurs from the interplay between CGS and TS, two enzymes that compete for a common substrate, OPH (Giovanelli et al., 1980). In wild type plants the level of Met and its metabolite SAM, control the partitioning of OPH. SAM activates TS (Curein et al., 1998), and Met or one of its metabolites represses CGS expression (Thompson et al., 1982; Chiba et al., 1999; Fig. 1). The finding reported here, that transcriptional upregulation of CGS increases the soluble Met and SMM level in Arabidopsis supports this hypothesis. Yet, CGS overexpression did not cause Thr to decline in proportion to the increase in Met and SMM, as might have been expected if CGS and TS compete for a fixed pool of OPH. This result suggests that the OPH pool is not fixed and that the rate of OPH synthesis may change in response to increased OPH utilization by CGS.

The morphologies of CGS and SAMS silenced plants are remarkably similar, suggesting that the growth abnormalities are due in part to a reduced ability to produce SAM. Similar morphological aberrations are produced in Arabidopsis by inhibition of CGS expression through antisense RNA (Kim and Leustek, 2000). Both Met and SAM play central roles in plant metabolism. SAM is the precursor of ethylene and certain polyamines, and it serves as the methyl group donor in DNA methylation reactions. Ethylene and polyamines are known to play prominent roles in growth, flowering and fruit development so disruption of these processes by CGS and SAMS cosuppression is expected.

An interesting property of plants showing CGS or SAMS cosuppression is the large scale, global increase in all amino acids. Amino acids also accumulate in Arabidopsis where CGS level is reduced by expression of antisense RNA (Kim and Leustek, 2000). The only major difference between the lines is that SAMS silenced plants accumulate Met and SMM, whereas CGS silenced plants do not (Fig. 4). There likely are multiple reasons for the increase in amino

acids. The first possibility is that a block in translation causes free amino acids to accumulate. Similar global increases in amino acids occur when a variety of amino acid biosynthetic enzymes are blocked with inhibitors or through antisense RNA techniques (Guyer et al., 1995; Kim and Leustek, 2000). A second possibility is that CGS or SAMS silencing causes the rate of amino acid synthesis to increase. Guyer et al., (1995) found that the expression of certain amino acid biosynthesis enzymes is increased in Arabidopsis when starved for a single amino acid. They proposed that Arabidopsis might contain a general amino acid control system analogous to the one regulated by the GCN4-transcription factor in yeast. Whether the loss of CGS or SAMS causes the rate of amino acid synthesis to increase has not yet been studied.

SAMS silenced plants accumulate 250 fold more Met than wild type (Fig. 4). This level is much higher than the 3 to 5 fold accumulation of other amino acids, indicating that Met-specific processes have been activated in the SAMS silenced plants. There are several possible explanations for the increase in Met. The loss of SAMS would be expected to eliminate a major route for Met metabolism. Also, the rate of Met synthesis would be expected to increase for the following reasons. (1) TS is allosterically activated by SAM (Curien et al., 1998), thus TS activity is probably reduced in SAMS silenced plants due to the likely reduction in SAM level; (2) CGS expression is increased by 3 fold (Fig. 5); and (3) the level of OPH is greater in SAMS silenced plants (not shown). All of these effects would allow increased ability of CGS to direct OPH toward Met.

The pleiotropic effect of SAMS silencing on CGS expression is particularly intriguing because CGS level is increased despite the accumulation of extremely high Met levels. Previous results showed that application of Met to living plants causes a decline in CGS level (Thompson et al., 1982; Chiba et al., 1999). However, in these studies it was not clear whether CGS repression was caused by Met or one of its metabolites, e.g. SAM. The present result suggests that SAM, rather than Met, is probably the negative regulator of CGS expression. If so, it is tempting to speculate that SAM is the factor that mediates autogenous control of CGS expression mediated through the MTO1 region.

적 요

메티오닌과 아데노실메티오닌 생합성에서 첫 단계는 시스타티온 감마-합성효소 (CGS)에 의해 촉매되어진다. 35S 발현조절부위의 조절 하에서 CGS를 대량발현하는 형질전환 애기장대는 발달의 특정 시기에만 메티오닌과 그 대사산물인 메틸메티오닌이 증가를 보여준다. CGS를 대량발현하는 어린 식물체는 에티오닌에 저항성이 있다. 유사한 결과들이 애기장대 CGS를 대량발현하는 형질전환 감자 식물체들에서 얻어졌다. 여러개의 형질전환체들에서 안티센스 RNA (CGS[-])에 의한 형질전환체와 비슷한 번식생장에서 감소된 능력을 가진 기형적인 식물들을 결과하는 CGS의 유전자 침묵을 보여준다. CGS[-] 와 CGS[+] 침묵인 형질전환식물체에 메티오닌의 외부적 첨가는 그들의 성장을 부분적으로 재생시킨다. 유사한 표현형적 기형들이 아데노실메티오닌 합성효소 (SAMS) 가 유전자 침묵된 식물들에서 관찰되었는데, 이들은 자연형 메티오닌 함량보다 250배 이상 축적하고도 CGS를 대량발현한다. 이 결과들은 CGS와 SAMS 침묵에 관련된 기형들이 부분적으로 아데노실메티오닌을 생산하는 감소된 능력 때문이며 아데노실메티오닌이 CGS 발현의 조절자임을 암시한다.

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